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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of: ROSENTHAL et al.	Group Art Unit: 1614
Serial No.: 10/728,277	Examiner: Roberts, Lezah
Filed: December 4, 2003	RULE 132 DECLARATION OF GARY J. ROSENTHAL (37 C.F.R. § 1.132)
Conf. No.: 7142	
Atty. File No.: 42830-10010	
For: "TREATMENT OF MUCOSITIS"	

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir or Madam:

I, Gary J. Rosenthal, residing at 11255 Dobbins Run, Lafayette, CO 80026, declare as follows:

I. Qualifications And Basis For Declaration:

I am a named inventor on U.S. Patent Application No. 10/728,277 (the "**Pending Application**"), which was assigned to RxKinetix, Inc., now renamed Endo Pharmaceuticals Colorado, Inc. I am not currently employed by Endo Pharmaceuticals Colorado, Inc., but am engaged by Endo Pharmaceuticals Colorado, Inc. as a paid technical consultant. Through my work with Endo Pharmaceuticals Colorado, Inc., I have considerable experience concerning oral mucositis as a side effect of cancer therapy. I also have considerable experience in general in the area of pharmacology, drug delivery and toxicology. I am board certified in General Toxicology by the American Board of Toxicology. The attached **Appendix A** is a detailed summary of my technical qualifications.

I have reviewed and considered an Office Action dated January 5, 2007 issued by the United States Patent and Trademark Office (the "**Office Action**") concerning the Pending Application, a copy of which is included in **Appendix B**.

I have reviewed and considered the following U.S. patent references cited in the Office Action, a copy of each of which is included in **Appendix C**:

1. U.S. Patent Number 4,188,373 by Krezanoski ("**Krezanoski**");
2. U.S. Patent Number 5,358,705 by Boggs et al. ("**Boggs et al.**"); and
3. U.S. Patent Number 5,861,174 by Stratton et al. ("**Stratton et al.**").

I have reviewed and considered the representative claims that are set forth in **Appendix D**.

Copies of the following technical papers, which I have reviewed and considered and to which I make reference below, are included in **Appendix E**:

1. Liao, YH et al. Biological Monitoring of Exposures to Aluminum, Gallium, Indium Arsenic and Antimony in Optoelectronic Industry Workers. JOEM. 2004. Vol 46. pp 931-936 ("**Liao et al.**");
2. Blair, HC et al. Reversible Inhibition of Osteoclastic Activity by Bone-Bound Gallium (III). J Cell Biochem. 1992. Vol 48. pp 401-410 ("**Blair et al.**");
3. Alfrey, AC et al. The Dialysis Encephalopathy Syndrome: Possible Aluminum Intoxication. NEJM. 1976. Vol 294. pp 184-188 ("**Alfrey et al.**"); and
4. Campbell, A et al. Differential Toxicity of Aluminum Salts in Human Cell Lines of Neural Origin: Implications for Neurodegeneration. Neuro Toxicology. 2001. Vol 22. pp 63-71 ("**Campbell et al.**").

This declaration concerns in particular the rejections of claims made in the Office Action 1) based on Krezanoksi in view of Boggs et al. and 2) based on Boggs et al. in view of Stratton et al.

II. Excerpts Of Teachings Of Krezanoski, Boggs et al. and Stratton et al.

For ease of reference to various portions of the teachings of Krezanoski, Boggs et al. and Stratton et al., the following excerpts from those references are provided below and assigned

reference numbers. In the discussions that follow in sections III and IV of this Declaration, I make reference as appropriate to various ones of these excerpts to identify representative portions of the document in relation to my opinions on the teachings of those documents. References to these excerpts are made in brackets in the body of the discussion below using the assigned reference numbers.

A. Excerpts From Krezanoski.

The following are some excerpts from Krezanoski (each identified by a reference number beginning with "K"):

K1. Column 2, line 49 through column 3, line 19 (emphasis added).

> In its broadest sense, the present invention provides a pharmaceutical vehicle useful for delivering a compatible, pharmacologically active chemical, i.e., drug or medicament, to a mucous membrane which consists of a clear, water-miscible, physiologically-acceptable, liquid composition which gels to a thickened, non-flowing and adhering consistency at human body temperature. Pharmaceutical vehicles in accordance with the invention are liquid at ambient room temperatures below about 30° C., preferably about 25° C. and below. They have a sol-gel transition temperature in the range of from about 25° C. to about 40° C., preferably from about 25° C. to about 35° C., and most preferably from about 29° C. to about 31° C.
>
> In accordance with the present invention, it has been discovered that aqueous solutions of certain polyoxyethylenepolyoxypropylene block copolymers are useful pharmaceutical vehicles having the properties set forth above. In particular, the present invention provides a pharmaceutical vehicle or base for carrying a pharmaceutically active material, i.e., a drug or medicament, which comprises:
>
> (a) from about 10% to about 26%, preferably from about 17% to about 26%, by weight of a polyoxyethylene-polyoxypropylene block

copolymer in which the number of polyoxyethylene units is at least about 50%, preferably about 70%, of the total number of monomeric units in the total molecule, the block copolymer having an average molecular weight of from about 7500 to about 15,500, preferably about 11,500, a room temperature solubility in water of greater than about 10 grams per 100 ml. of water, and a cloud point in 1% aqueous solution of at least about 100° C.; and

(b) from about 74% to about 90% by weight water, the vehicle having a sol-gel or gel transition temperature in the range of from about 25° C. to about 40° C., preferably from about 25° C. to about 35° C., and especially from about 29° C. to about 31° C.

K2. Column 3, lines 37-46 (emphasis added).

In keeping with the concept of the present invention, there is also provided a pharmaceutical composition useful for protective or therapeutic application to mucous membranes comprising a solution of a pharmacologically effective amount of a pharmaceutically active material, i.e., drug or medicament, in a pharmaceutical vehicle as described above. The concept of this invention is not dependent on the nature of the drug, and any compatible pharmaceutically active material may be used.

K3. Column 3, line 61 through column 4, line 3 (emphasis added).

An important aspect of this invention is that the pharmaceutical vehicles and compositions are liquid at ambient room temperatures and can be applied to the affected mucous membrane area by conventional liquid depositing means, including dispensation to the area of treatment from standard plastic squeeze bottles or in drop form. At body temperatures above 30° C., the vehicle or base passes through the sol-gel transition temperature and gels to a thickened, non-flowing and adhering

consistency, holding and delivering the medication as required and for prolonged periods of time.

K4. Column 5, lines 43-53.

It has been found that a useful block copolymer concentration is from about 10% to about 26% by weight, particularly from about 17% to about 26%. Excellent results have been obtained using aqueous solutions of from about 17% to about 26% by weight of "Pluronic F-127". The water content is generally from about 74% to about 90% by weight of the vehicle composition, and is typically from about 74 to about 85% by weight. The water used in forming the aqueous solution is preferably purified, as by distillation, filtration, ion-exchange or the like.

K5. Column 5, lines 54-61 (emphasis added).

The polyoxyethylene-polyoxypropylene pharmaceutical vehicles of this invention have been unexpectedly found to increase drug absorption by the mucous membrane. Moreover, it has also been found that the pharmacologic response is unexpectedly prolonged. Drug action is typically both increased and prolonged by a factor of 2 or more. At the same time, protection is afforded to the involved tissues.

K6. Column 7, lines 30-39 (emphasis added).

Any pharmaceutically active material may be admixed in a pharmacologically effective amount with the pharmaceutical vehicle to form the pharmaceutical compositions of this invention. Preferably, the drug is water-soluble. However, drugs which are not ordinarily soluble in water may also be employed, and it has been found that a wide variety of useful drugs which are currently marketed in suspension form can be

dissolved in the polyoxyethylene-polyoxypropylene vehicles of the present invention.

K7. Column 8, lines 20-24 (emphasis added).

In addition to overcoming major disadvantages of previous techniques for delivering drugs and medicaments to mucous membranes, the present invention has been found to increase drug absorption by the affected tissue and prolong pharmacologic response.

B. Excerpts From Boggs et al.

The following are some excerpts from Boggs et al. (each identified by a reference number beginning with "B"):

B1. Column 1, lines 44-51 (emphasis added).

The subject invention encompasses compositions for reducing or preventing dental plaque, or gingival or periodontal diseases, of the oral cavity in humans or lower animals comprising a safe and effective amount of a complex of metal ion with N-acetylated amino acid wherein the metal ion is selected from the group consisting of In^{3+}, Ga^{3+}, and Al^{3+}; and a pharmaceutically-acceptable carrier.

B2. Column 1, lines 55 through column 2, line 11 (emphasis added).

The compositions of the subject invention comprise a complex of a metal ion with one or more N-acetylated amino acids in a pharmaceutically-acceptable topical oral carrier.

"Pharmaceutically-acceptable topical oral carrier", as used herein, denotes a carrier for the active compound or compounds of the subject invention (hereinafter "Active" or "Actives") comprising solid or liquid

filler diluents suitable for use in contact with the oral tissues of humans and lower animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio. Such topical oral carrier, when combined with an Active, results in a composition which is administered topically to the oral cavity. Preferably such compositions are held in the oral cavity for a period of time, and then largely expectorated rather than being swallowed. Such compositions include mouthwashes, mouth rinses, mouth sprays, dental treatment solutions, toothpastes, dental gels, tooth powders, prophylaxis pastes, lozenges, chewing gums and the like and are more fully described hereinafter. Dentifrices and mouthwashes are the preferred compositions.

By the term "metal ion", as used herein, is meant Al^{3+}, Ga^{3+}, or In^{3+}.

B3. Column 2, line 48 through colmn 3, line 2 (emphasis added).

The subject invention provides a plaque inhibiting composition comprising certain Actives which are complexes of metal ions with N-acetylated amino acids. The ratio of metal ion to N-acetylated amino acid is preferably from about 1:4 to about 1:1, more preferably from about 1:3 to about 1:1.

While not limited to such structures, the metal ion/N-acetylated amino acid complex is believed generally to conform to the following structure:

$R_nM_3(OH)_{9-n}$

In the above structure, each M is independently a metal selected from the group consisting of indium, gallium, and aluminum. Preferred is all M being the same; more preferred is all M being aluminum.

In the above structure, each R is independently selected from N-acetylated amino acids. Preferably R is an N-acetylated naturally

occurring amino acid; more preferably R is an N-acetylated naturally occurring L-amino acid; more preferably still R is N-acetyl-L-glutamine or N-acetyl-L-cysteine. Preferred is all R being the same.

B4. Column 3, lines 21-33 (emphasis added).

The Actives of the subject invention are soluble in conventional oral care compositions, such as mouthwashes, mouth rinses, and toothpastes. However, oral care compositions of the subject invention should be substantially free from materials known to complex strongly with aluminum, gallium, or indium or to form insoluble precipitates with those metal ions. Materials to avoid include fluoride ions, phosphate ions, metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetic acid (NTA), and others. Inclusion of substantial quantities of these substances in a composition tends to negate the efficacy of the Actives and to reduce anti-plaque activity.

B5. Column 3, lines 34-58 (emphasis added).

It has been unexpectedly found that treatment of teeth with Actives of the subject invention leads to a dramatic reduction in bacteria binding to the tooth surface. While the anti-plaque activity of the Actives is not limited by the following, it is believed that such activity is at least partially achieved in the manner described hereinbelow. The hydroxyapatite surfaces of teeth possess phosphate and calcium receptors. The Actives possessing metal ions of aluminum, gallium or indium bind to the phosphate receptors while displacing some or all of the N-acetylated amino acid components of the Actives. The N-acetylated amino acid component of the Active, being more weakly complexed to the metal ion, is displaced by the phosphate group attached to the hydroxyapatite surface. The result appears to be a metal phosphate coating on the

hydroxyapatite surface. Hydroxy groups and water appear to be bound to the metal ions forming a hydrophilic hydrous gel layer. The gel layer inhibits bacteria from obtaining access and binding to saliva proteins in the pellicle on the tooth surface. Because bacteria are impeded from adhering to the teeth, fewer are present on the tooth surface to multiply. The result is a reduction in bacterial accumulation, therefore in plaque and gingivitis when used in the oral cavity.

B6. Column 4, lines 19-32 (emphasis added).

By "safe and effective amount" as used herein is meant an amount of compound or composition sufficient to induce a significant positive modification in the condition to be treated, but low enough to avoid serious side effects (at a reasonable benefit/risk ratio), within the scope of sound medical judgment. The safe and effective amount of the compound or composition will vary with the particular condition being treated, the age and physical condition of the patient being treated, the severity of the condition, the duration of the treatment, the nature of concurrent therapy, the specific compound or composition employed, the particular pharmaceutically-acceptable carrier utilized, and like factors.

B7. Column 4, lines 36-50 (emphasis added).

Components of the topical, oral carrier are suitable for administration to the oral cavity of a human or lower animal and are compatible with one another and the other components, especially the Active, used in an oral composition of the subject invention . . . Preferred topical, oral carriers thus provide the desired characteristics for mouthwashes, mouth rinses, mouth sprays, dental treatment solutions, toothpastes, dental gels, toothpowders, prophylaxis pastes, lozenges, chewing gums, and the like.

C. Excerpts From Stratton et al.

The following are some excerpts from Stratton et al. (each identified by a reference number beginning with an "S"):

S1. Column 1, line 36 through column 2, line 17 (emphasis added).

> Traditionally, the most widely used method of administration of therapeutic agents is by the oral route. However, such delivery is not feasible, in the case of macromolecular drugs, as they are rapidly degraded and deactivated by hydrolytic enzymes in the alimentary tract. Even if stable to enzymatic digestion, their molecular weights are too high for absorption through the intestinal wall. Consequently, they are usually administered parenterally; but, since such drugs often have short half-lives in vivo, frequent injections are required to produce an effective therapy. Unfortunately, while the parenteral route is the most efficient means of drug introduction, this route has severe drawbacks in that injections are painful; they can lead to infection; and they can lead to severe vascular problems as a result of repeated intravenous injections.
>
> For these reasons, biodegradable polymer matrices have been considered as sustained release delivery systems for a variety of active agents or drugs. Once implanted, the matrix slowly dissolves or erodes, releasing the drug. An alternative approach is to use small implantable pumps, which slowly extrude the drug and matrix components, which dissolve after contacting body fluids. With both systems it is crucial that the drug remain evenly distributed throughout the matrix since heterogeneous distribution of the drug (e.g., formation of large clumps and voids) could lead to erratic dosing. Furthermore, both systems require polymers that remain somewhat fluid so that they can be easily manipulated prior to implantation or loading into a device.
>
> The use of polymers as solid implants and for use in small

implantable pumps for the delivery of several therapeutic agents has been disclosed in scientific publications and in the patent literature. [Citations omitted.] However, the polymers having the greatest potential for use in the delivery of protein drugs would exhibit reverse thermal gelation and have good drug release characteristics.

S2. Column 2, line 56 through column 3, line 18.

While poloxamers, and more specifically Pluronic® F-127 or Poloxamer 407, have been used to deliver nonpeptidic drugs as well as biologically active proteins, [citations omitted] sustained delivery of biologically active macromolecules for weeks or months has not been possible for reasons that are two-fold. First, previous references which disclose the incorporation of proteins in a Pluronic® matrix only disclose solutions of a protein, with concentrations less than approximately 2 mg/ml and second, formulation approaches used to incorporate proteins into polymeric systems often result in irreversible inactivation of the proteins because of the presence of organic solvents, pH changes, and thermal effects. Consequently, prior references which teach the use of poloxamers as pharmaceutical vehicles for the delivery of proteins have suffered two serious limitations; (i) low initial concentrations of protein are used, and (ii) an unacceptable percentage of the protein loses its biological activity during use or storage. These two limitations have a direct impact on the ability to produce a polypeptide drug delivery system which can be shelved for long periods of time prior to usage and administer controlled dosages of protein for a period of weeks or more preferably months. Furthermore, degraded proteins can have reduced efficacy as a drug, and can also elicit adverse reactions, such as sensitization and adverse immune response.

There is still a need, therefore, for a polypeptide drug delivery device or composition having high concentrations of fully native

macromolecular polypeptides which may be regularly released over a long period of time.

S3. Column 3, lines 21-45 (emphasis added).

Accordingly, it is an object of this invention to provide a polypeptide drug delivery system . . . To achieve the foregoing and other objects and in accordance with the purposes of the present invention, as embodied and broadly described therein, the composition of the present invention comprises a polymeric matrix having thermal gelation properties in which is incorporated a suspension of at least one biologically active macromolecular polypeptide having a concentration of 0.5 percent or greater by weight of the composition.

S4. Column 3, line 64 through column 4, line 18 (emphasis added).

The pharmaceutical device or composition of the present invention provides a delivery system for the controlled and sustained administration of fully native macromolecular polypeptides or therapeutic agents to a human or animal. The biodegradable, biocompatible matrix for drug delivery is formed by suspending soluble and insoluble particles of fully native macromolecular polypeptides having a concentration of 5 mg/ml or greater, and other protein stabilizing components uniformly and discretely throughout a pharmaceutical vehicle or polymer matrix that exhibits reverse thermal gelation characteristics. As with previously known systems, the suspended particles and other components are released through a combination of diffusion and dissolution mechanisms as the device hydrates and subsequently erodes or dissolves. However, unlike known polymeric matrix systems which deliver macromolecules, the composition of this invention comprises a suspension as opposed to a solution of native polypeptide(s). Consequently, high polypeptide

concentrations are attained as a result of the suspension, thus achieving the ability for sustained administration of the therapeutic agent for a time period of days, weeks or months as opposed to hours.

S5. Column 4, lines 45-62.

In order to study protein structure with infrared spectroscopy it is necessary to have protein concentrations of at least 15-20 mg/ml, thus a protein concentration of 20 mg/ml was prepared in the presence of sufficient poloxamer to allow gelling during warming. The resulting protein solution formed a fine, milky suspension, which was initially very disappointing, because the formation of such suspensions often indicates that a solution component (e.g., the polymeric detergent) caused the protein to denature and to form non-native or inactive protein aggregates, thus indicating failure of the test. However, infrared spectroscopy can, fortunately, be used to analyze protein structures in suspension, so the suspension was analyzed anyway. Surprisingly, when the protein suspension was analyzed with the Fourier transform infrared spectroscopy, instead of finding the expected non-native or inactive protein aggregates, it was unexpectedly found to be fully native.

S6. Column 5, lines 5-19.

A preferred polyoxyalkylene block copolymer for use as the pharmaceutical vehicle of this invention is a polyoxyethylene-polyoxypropylene block copolymer having the following formula . . . the polyoxyethylene chain constituting about 70 percent of the total number of monomeric units in the molecule and where the copolymer has an average molecular weight of about 12,600. Pluronic® F-127, also known as Poloxamer 407, is such a material.

S7. Column 5, lines 35-45 (emphasis added).

Any macromolecular polypeptide may be mixed with the pharmaceutical vehicle to form the pharmaceutical composition of this invention wherein the concentration of macromolecular polypeptide is in the range of 0.5 to 50 percent by weight of the composition. The choice of polypeptides which can be delivered in accordance with the practice of this invention is limited only by the requirement that they be at least very slightly soluble in an aqueous physiological media such as plasma, interstitial fluid, and the intra and extracellular fluids of the subcutaneous space and mucosal tissues.

S8. Column 5, line 61 through column 6, line 10 (emphasis added).

The pharmaceutical composition of the present invention can be readily prepared using any solution forming technique which achieves the concentration of polyoxyalkylene block copolymer necessary for gelling. Preferably the pharmaceutical vehicle and polypeptide mixture are prepared separately and the polypeptide mixture having a concentration of 5 mg/ml or greater is added thereto at a temperature of about 0° C. to 10° C. When combined the protein forms a homogenous suspension of fine particles in the polymer solution, which then has a "milky" appearance. By light microscopy the particles are approximately 5-10 microns in diameter. Raising the sample temperature above the gel point of the poloxamer results in an even distribution of protein particles throughout the polymer gel. Due to the high viscosity of the gel matrix, the particles remain homogeneously distributed and do not "settle out." The liquid to gel transition is fully reversible upon cooling.

S9. Column 6, lines 15-20 (emphasis added).

> The pharmaceutical composition of the present invention can be implanted directly into the body by injecting it as a liquid, whereupon the pharmaceutical composition will gel once inside the body. In the alternative, the pharmaceutical composition may be introduced into a small implantable pump which is then introduced into the body.

S10. Column 6, lines 35-45.

> It is known that a gel will not form when the concentration of polyoxyethelene-polyoxypropylene block copolymer in water or dilute buffer is outside of the range of about 20 to 30 percent by weight, as shown in FIG. 1 and exemplified by the line having open triangles. However, by introducing protein-stabilizing solutes to the pharmaceutical device of the present invention the gel-sol transition temperature may be manipulated, while also lowering the concentration of polyoxyethelene-polyoxypropylene block copolymer which is necessary to form a gel.

III. Krezanoski In View Of Boggs et al.

I have the following opinions in relation to the teachings of Krezanoski and Boggs et al. and the rejection of claims stated in the Office Action based on Krezanoski in view of Boggs et al.:

Krezanoski discloses a drug delivery vehicle (referred to as a "pharmaceutical vehicle") targeted for delivering a drug (referred to as a "pharmaceutically active chemical" or "pharmaceutically active material") to mucous membranes [K1, K2, K3]. Krezanoski reports that the drug delivery vehicle was found to enhance absorption of the delivered drug by the mucous membrane [K5, K7]. The delivery vehicle of Krezanoski is intended to be general purpose for mucosal delivery applications, in that Krezanoski discloses that the invention is not dependent on the nature of the drug [K2] and that any pharmacologically active material may be admixed in the vehicle [K6].

Boggs et al. disclose a composition for treating dental plaque, or gingival or periodontal diseases, of the oral cavity [B1, B2]. The composition is designed for and treatment is targeted for topical delivery in the oral cavity [B2, B4, B7] of an active component (referred to as an "Active" [B2]), which is a complex of aluminum, gallium or indium metal ion with N-acetylated amino acid [B1, B2, B3], for surface treatment of the teeth [B5]. Boggs et al. postulate that at the hydroxyapatite surface of the teeth, the metal ion of the active component binds with phosphate receptors present at the tooth surface, with the N-acylated amino acid of the active component being displaced by the phosphate group, the result of this surface treatment being the formation of a metal phosphate coating on the teeth that forms a protective layer that inhibits bacteria from accessing the teeth, leading to a reduction in bacterial accumulation and therefore also a reduction in plaque and gingivitis [B5]. As described by Boggs et al., the only function of the N-acetylated amino acid is to act as a complexing agent that weakly complexes with the metal ion to keep the metal ion in solution until the metal ion reaches a tooth surface where the metal ion can dissociate from the N-acetylated amino acid to bind with phosphate receptors at the tooth surface [B5].

The only use of N-acetylcysteine disclosed in either Krezanoski or Boggs et al. is in Boggs et al. for use as the N-acetylated amino acid component to weakly complex with the metal ion component of the Active [B3]. Therefore, the only context of drug delivery for N-acetylcysteine disclosed by either Krezanoski or Boggs et al. is for the noted surface treatment of the teeth disclosed by Boggs et al.

The targeted drug delivery mechanisms, and problems associated with such mechanisms, disclosed by Krezanoski and Boggs et al. are fundamentally different, i.e., mucosal delivery to a mucous membrane in the case of Krezanoski and surface treatment of the teeth in the case of Boggs et al., and there is no apparent reason why a medical professional would even consider Krezanoski and Boggs et al. together, because they do not concern similar problems associated with drug delivery (effective absorption of a drug by the mucous membrane in the case of Krezanoski vs. effective binding of a drug to the surface of a tooth in the case of Boggs et al.). Rather, the medical professional would realize that the use of a delivery vehicle that increases absorption of drug by a mucous membrane (as in Krezanoski) would, absent some other special considerations, generally be inappropriate for use in the very different context where drug delivery is to be targeted for surface treatment of the teeth (as in Boggs et al). All else being

equal, for any given drug delivery composition introduced into a patient's oral cavity, the more of the drug that is lost to absorption by the mucous membrane in the oral cavity, the less of the drug that is potentially available for the desired surface treatment of the teeth. To counteract the loss of drug to mucosal absorption, a larger quantity of the drug would then be required for the desired surface treatment of the teeth. The selection of a drug delivery vehicle that would increase the quantity of drug required for the surface treatment of the teeth would generally be undesirable. Moreover, with any pharmacological treatment, it is generally desirable, all else being equal, to target the drug to the desired treatment location and minimize contact with bystander cells not targeted for treatment, there being a general desire to minimize the potential for side effects on the bystander cells. Absent the presence of some other special considerations that would counteract these general considerations, it would be undesirable from a pharmacological perspective when targeting a drug to a surface treatment of the teeth to select a drug delivery vehicle that would increase absorption of that drug by the mucous membrane. Neither Krezanoski nor Boggs et al. disclose any such other special considerations.

However, in relation to the surface treatment of the teeth that is the focus of Boggs et al., there is an additional consideration that would further dissuade a medical professional from combining the delivery vehicle of Krezanoski with the metal ion complex with N-acetylcysteine of Boggs et al. This additional consideration is that all of the metals specified by Boggs et al. for use in the complex (aluminum, gallium and indium) have been identified as having significant potential for toxicity at elevated concentrations in the human body. Reference is made generally to Liao et al., Blair et al., Alfrey et al. and Campbell et al. simply as examples of some of the work that has been performed in relation to the toxicity of these metals.

Boggs et al. recognize that there is a benefit/risk analysis associated with providing sufficient drug for effective treatment, but low enough to avoid serious side effects, within the scope of sound medical judgment [B6]. Based on disclosures of Krezanoski and Boggs et al., and in view of sound medical judgment, a medical professional wanting to perform a surface treatment of the teeth using the metal ion complex with N-acetylcysteine as disclosed by Boggs et al., would not use the delivery vehicle of Krezanoski, because of the disclosure by Krezanoski that that delivery vehicle was found to increase drug absorption by the mucous membrane, for at least the following reasons:

1. Increased absorption of the metal ion by the mucous membrane in the oral cavity would reduce the amount of the metal ion active that would be available for the desired binding of the metal ion at the surface of the patient's teeth.

2. The metals used as actives by Boggs et al. are known to have significant risks of toxicity, and increased absorption of the metal ion active by the mucous membrane in the oral cavity would unnecessarily increase risk of toxic exposure of the patient to those metals, and there is no possible apparent benefit to be obtained by such increased absorption of the metal ion active by the mucous membrane that could justify an increased risk of toxic exposure, because the targeted treatment by Boggs et al. is based on a surface effect occurring at the surface of the teeth that would not be promoted by increased absorption of the metal ion active by the mucous membrane.

Additionally, there is nothing in Krezanoski or Boggs et al. that would suggest that the therapeutic composition recited in Claim 1 set forth in Appendix D would possess properties beneficial for efficacious treatment of oral mucositis as a side effect of cancer therapy, and such properties would be unexpected from the disclosures of Krezanoski and Boggs et al.

IV. Boggs et al. In View Of Stratton et al.

I have the following opinions in relation to the teachings of Boggs et al. and Stratton et al. and the rejection of claims stated in the Office Action based on Boggs et al. in view of Stratton et al.:

As discussed above, Boggs et al. disclose a composition for treating dental plaque, or gingival or periodontal diseases, of the oral cavity, with the treatment targeted for delivering an aluminum, gallium or indium metal ion active to the teeth for surface treatment of the teeth.

The teachings of Stratton et al. are directed to addressing problems with the sustained systemic delivery through the parenteral route of macromolecular drugs, and particularly macromolecular polypeptides such as proteins [S1, S2]. To address these problems, Stratton et al. disclose a composition for use as a polypeptide drug delivery system, that is in the form of a

polymeric matrix having thermal gelation in which is incorporated a suspension of a biologically active, macromolecular polypeptide at a relatively large concentration (0.5 percent or greater) [S3, S4]. The polypeptide drug delivery system is designed for systemic delivery of polypeptides over a prolonged period, such as through implantation into the body by injection or by introduction into a small implantable pump introduced into the body [S9], and the polypeptide delivery system is disclosed as a general purpose delivery vehicle for such sustained delivery applications, in that the delivery vehicle is disclosed for use with any macromolecular polypeptide that is at least very slightly soluble in an aqueous physiological media [S7].

Consistent with its design for sustained/controlled release applications, the composition of Stratton et al. is targeted for parenteral administration. The background section of Stratton et al. discusses the desirability of parenteral administraton [S13], and Stratton et al. specifically disclose as delivery routes only injection into the body as a liquid that gels once inside the body or, alternatively, delivery from a small implantable pump that is introduced into the body [S7].

As was the case with Krezanoski and Boggs et al. discussed above, so also the targeted drug delivery mechanisms, and problems associated with such mechanisms, disclosed by Boggs et al. and Stratton et al. are fundamentally different, i.e., surface treatment of the teeth in the case of Boggs et al. and sustained systemic delivery through parenteral placement in the case of Stratton et al., and there is no apparent reason why a medical professional would even consider Boggs et al. and Stratton et al. together, because they do not concern similar problems associated with drug delivery (effective binding of a drug to the surface of a tooth in the case of Boggs et al. vs. effective parenteral placement and sustained release of a drug in the case of Stratton et al.).

Moreover, Stratton et al. address the issue of sustained delivery of polypeptides by proposing that the polypeptide delivery composition contain polypeptides in a stable form as precipitates held in a polymer matrix [S2, S5, S8], whereas Boggs et al. address the issue of delivery of drug for surface treatment of the teeth quite differently through the use of a weakly associated complex of an active metal ion with an N-acetylated amino acid [B1, B5]. Delivery of the metal ion of Boggs in the form of a precipitate held in a polymer matrix as used by Stratton et al. would be counterproductive to the nature of the surface treatment targeted by Boggs et al, because the precipitate and polymer matrix of Stratton et al. would tend to reduce the availability of the metal ion to access the targeted phosphate receptors at the tooth surface for

the desired binding to phosphate receptors at the tooth surface. In fact, Boggs et al. specifically teach that the formation of insoluble precipitates of the metal ions is undesirable [B4].

Again, the only mention of N-acetylcysteine is in Boggs et al. for use as a complexing agent for the metal ion directed to a surface treatment of the teeth. Given the fundamentally different drug delivery contexts of Boggs et al. and Stratton et al., a medical professional would find no reason to select the polymeric matrix drug delivery vehicle of Stratton et al. for the surface treatment application of Boggs et al. Moreover, consistent with the discussion above concerning Krezanoski, there would be some strong considerations that would positively dissuade a medical professional from making such a combination.

Both Stratton et al. and Krezanoski et al. use the same types of polyoxyethylene-polyoxypropylene block copolymers, with a preferred polymer being Pluronic F-127 [K1, K4, S2, S6] (the same type of polymer as specified in Claim 1 of Appendix D), formulated at polymer concentrations so that the drug delivery composition in each case exhibits reverse thermal gelation [K1, K3, K4, S8, S9, S10]. Also, Stratton et al. use Pluronic F-127 in all of the examples presented in their patent, and Krezanoski uses Pluronic F-127 in seven of the eight examples presented in his patent. Because of these similarities in the drug delivery vehicles based on polyoxyethylene-polyoxypropylene of Krezanoski and Stratton et al., a medical professional would expect that if the drug delivery vehicle of Stratton et al. were used for topical oral administration, that it would also exhibit the property of increased drug absorption by the mucous membrane that was disclosed to be found by Krezanoski. Therefore, consistent with the discussion above, based on the disclosures of Krezanoski, Stratton et al. and Boggs et al., and in view of sound medical judgment, a medical professional wanting to perform a surface treatment of the teeth using the metal ion complex with N-acetylcysteine as disclosed by Boggs et al., would not use the delivery vehicle of Stratton et al., because of the disclosure by Krezanoski that that delivery vehicle was found to increase drug absorption by the mucous membrane, for at least the following same reasons as noted above:

1. Increased absorption of the metal ion by the mucous membrane in the oral cavity would reduce the amount of the metal ion active that would be available for the desired binding at the surface of the patient's teeth.

2. The metals used as actives by Boggs et al. have known risks of systemic toxicity and increased absorption of the metal ion active by the mucous membrane in the oral cavity would unnecessarily increase risk of toxic exposure of the patient to those metals, and there is no possible apparent benefit to be obtained by such increased absorption of the metal ion active by the mucous membrane that could justify an increased risk of toxic exposure, because the targeted treatment by Boggs et al. is based on a surface effect occurring at the surface of the teeth that would not be promoted by increased absorption of the metal ion active by the mucous membrane.

Additionally, there is nothing in Boggs et al. or Stratton et al. that would suggest that the therapeutic composition recited in Claim 1 set forth in Appendix D would possess properties beneficial for efficacious treatment of oral mucositis as a side effect of cancer therapy, and such properties would be unexpected from the teachings of Boggs et al. and Stratton et al.

All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. I understand that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of this patent application or any patent issuing thereon.

Respectfully submitted,

Date: 28 June 2007

By: _____
Gary J. Rosenthal

APPENDIX A

TO

DECLARATION

OF

GARY J. ROSENTHAL

Detailed Summary Of Technical Qualifications

Curriculum Vitae

GARY J. ROSENTHAL

CITIZENSHIP	United States
HOME ADDRESS	11255 Dobbins Run Lafayette, CO 80026

EDUCATION / CERTIFICATION

1979	**B.S. - Boston College** Double Major: Biology and Philosophy
1983	**M.S. - New York University** Institute of Environmental Medicine
1984	**Ph.D. - New York University** Institute of Environmental Medicine
1990	**American Board of Toxicology** • Board Certified in General Toxicology • Re-certified – 1995, 2000, 2005 • Executive Board of Directors, 1996-2000

EXPERIENCE

December 2005 – present	NOBLE MOLECULES, Boulder, CO Co-Founder, Sr. Vice President, Director, R&D

Start-up focused on identification and development of proprietary formulations and new uses for previously approved human pharmaceuticals.

October 1997 – October 2006	RxKINETIX, INC., Boulder, CO Vice-President, Drug Development RxKinetix, Inc. was acquired by Endo Pharmaceuticals, Inc., and its name was changed to Endo Pharmaceuticals Colorado, Inc., in Oct. 06 and currently serve as consultant to Endo Pharmaceuticals, Colorado, Inc., Oct 06 – present)

Worked with founders, CEO and management team to build functional infrastructure and raise venture capital to develop proprietary drug delivery technology and products targeting various diseases and through multiple routes of administration. Predominant focus on supportive oncology and infectious disease based indications. Active agents developed by the R&D efforts included antioxidants, hematopoeitic growth factors, and protein-based vaccines. Responsibilities included preclinical R&D, analytical development, and intellectual property. Responsible for collaborative efforts with academic and industrial partners.

April 1997 to October, 1997 TELOS, INC., Boulder, CO

Biopharmaceutical Consult

Summary of Experience - Biopharmaceutical and environmental safety consult. Due diligence for in-licensing opportunities. Established proprietary database of worldwide contract research facilities and efforts.

October 1992 to April 1997 SOMATOGEN, INC., Boulder, CO

Director - Pharmacology & Toxicology (6/93 - 4/97)
Sr. Toxicologist (10/92 - 5/93)

Responsible for preclinical research related to product development. Specific preclinical focus was on recombinant hemoglobin as a blood substitute and moving from IND to Phase 2 development. Research focus included blood and blood forming organs, metabolic, cardiovascular, renal and gastrointestinal systems. Oversight of pharmacology and toxicology operations including pharm/tox/ADME submissions to support regulatory requirements. Established many academic collaborations and was primary liason for rHb1.1 Pharm/Tox/Metabolism for ~2 years with corporate partner Eli Lilly.

1985 to 1992 NATIONAL INSTITUTES OF HEALTH/NIEHS, RTP, NC
National Toxicology Program, Systems Toxicity Branch
- Project Officer ('89 -'92)
- Tenured Scientist ('87 - '92)
- NIH Fellow ('85 - '87)

Conceive, plan, develop, conduct, oversee and contract applied research in the area of toxicology consistent with the interests of the National Institutes of Health and the National Toxicology Program. Mechanistic research related to environmental, pharmaceutical and industrial agents, as well as interpretation of toxicologic data and models of human disease. Experience in the generation of protocols, SOPs, and preparation of final reports. Supervisory experience with technical and support staff. Project Officer for NTP AIDS Drug toxicological contract studies. Specific focus on integrating cellular, biochemical, immunological and toxicological methods and data in safety assessment.

1980 - 1985 NEW YORK UNIVERSITY, NY, NY
Institute of Environmental Medicine
Laboratory of Inhalation Toxicology & Carcinogenesis
Pre-doctoral Inhalation Toxicology Fellow

Acute and sub-chronic testing protocols using various routes. Experience in quantitative analysis and technical report writing. Experience with whole body inhalation chambers including design and principals. Experience in the generation of atmospheres of dusts, vapors, and gases and techniques for measurement of such atmospheres.
Experience in toxicologic responses of pulmonary, hematopoeitic and immunologic systems.

1979 – 1980 NEW YORK UNIVERSITY, NY, NY
Cancer Research and Radiobiological Laboratory
Assistant Research Scientist

Radiation biology, experimental chemotherapy, and cellular biology protocols. Responsible for technical aspects of projects pertaining to radiation biology; cell culture dose-response protocols using chemotherapeutic agents; whole body animal irradiation; DNA alkaline elution.

1977 - 1979 BOSTON COLLEGE, Chestnut Hill, MA
Sontag Institute for Cancer Research
Undergraduate Research Fellow

Senior Research Thesis: "The Effects of Combined Chemotherapy Utilizing Lysosomal Labilizers and Methotrexate on Shay's Chloroma Tumor Bearing Mice."

EXECUTIVE BOARD OF DIRECTORS
American Board of Toxicology ('96-'00)
Elected Secretary ('98-'00)
Chairman, Recertification Committee ('97-'00)
VasoGenix (Founder / BoD member ('99 -'00)

PROFESSIONAL AFFILIATIONS:
Society of Toxicology
American Board of Toxicology
Controlled Release Society
American Assn. for the Advancement of Science

HONORS:
1980 – 1987 National Research Service Award (2)
1992 Public Health Service Performance Award
1994 Board of Publications Award / Best Paper in Fundamental and Applied Toxicology (co-author)

SERVED AS A REVIEWER FOR PUBLICATIONS IN:
Toxicology and Applied Pharmacology
Fundamental and Applied Toxicology
Environmental Health Perspectives
International Journal of Immunopharmacology
Environmental Research, Toxicology,
Cancer Research, Blood, Experimental Lung Research

American Journal of Respir. Cell and Molecular Biology

CONTINUING EDUCATION:

* Project Officer Training Course - NIH, 1991
* Development and Safety Evaluation of Recombinant Products for Pharmaceutical and Agricultural Use- SOT-1992
* NTP Health and Safety Training Course - NIH /1992
* TQM-University of Colorado 1994
* History, Theory and Trends in NP Management 2005, Regis University

PRINCIPAL ACADEMIC ADVISOR FOR:

Postdoctoral

Emanuela Corsini, University of Milan /NIH ('89-'92)

Mark Blazka , NIH Fellow ('91 - '92)

Pre-doctoral

Ling-Yuan Kong NIH/Univ. of Strathclyde (Scotland) ('91-'93)

PATENTS (Inventor/Co-inventor)

US Patent # 5,162,361 - Method of treating diseases associated with elevated levels of inflammatory cytokines

US Patent # 5,631,219 Method for Stimulating Hematopoiesis with Hemoglobin

US Patent # 6,809,079 - Compositions and methods for treating female sexual arousal disorder using hydrophobic-calcitonin gene related peptide

US Patent # 6,685,917 - Treatment of mucositis

US Patent # 6,649,189 - Methods for use of delivery composition for expanding, activating, committing or mobilizing one or more pluripotent, self-renewing and committed stem cells

US Patent # 6,875,441 Composition for delivery of hematopoietic growth factors

COMMITTEES:

- NIH/NIEHS AIDS Advisory Committee ('90-'92)
- NIAID Cmte for AIDS Drug Toxicology RFA's ('91-'92)
- NC-SOT Co-Chairman Education Committee (89-90)
- SOT Public Communications Committee ('91-'94)
- SOT/Immunotoxicology Specialty Section Awards Committee (1988, 1996)

Nominations and Program Committee (1989-90)
Regulations Committee ('91, '97)
• Councilor SOT / ITOX Specialty Section ('95-'97)
• NIH Technical Evaluation Committee – Microbial Screening of Animals for Microbial Pathogen (Cont. 273-89-P-0008)
• Peer Reviewer-Center for Indoor Air Research ('91 - present)

ADJUNCT FACULTY Colorado State University (1994-2001)

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BOOK CHAPTERS

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NATIONAL TOXICOLOGY PROGRAM TECHNICAL REPORTS AUTHORED

NTP Technical Report on the Immunotoxicity of dapsone (CAS No. 80-08-0): NTIS #PB92-140383 Vol 1: 2

NTP Technical Report on the Immunotoxicity of 2,3 dideoxydidehydrothymidine (D4T) (CAS No 3056-17-5): NTIS #PB92-140383 Vol 1: 4

NTP Technical Report on the Immunotoxicity of succinyl concanavalin A (SCA) (CAS No 55128-23-9): NTIS #PB92-140383 Vol 1; 14

INVITED SPEAKER

Immunomodulation by methotrexate in B-lymphocytes. 3rd Annual Duke Toxicology Retreat, Duke University Medical Center, Durham,N.C., May 1986

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APPENDIX B

TO

DECLARATION

OF

GARY J. ROSENTHAL

Copy of Office Action Dated January 5, 1007

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/728,277	12/04/2003	Gary L. Rosenthal	42830-10010	7142

25231 7590 01/05/2007

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EXAMINER	
ROBERTS, LEZAH	
ART UNIT	PAPER NUMBER
1614	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	01/05/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

Office Action Summary	Apolication No.	Applicant(s)	
	10/728 277	ROSENTHAL ET AL.	
	Examiner	Art Unit	
	Lexah V. Roberts	1614	

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C § 133)

Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *29 September 2006*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,15,17,19,20,22,24,25,31,35,38,133-137 and 142-148* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1, 15, 17, 19-20, 22, 24-25, 31, 35, 38, 133-137 and 142-148* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *A-B*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. ____
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

This office action is in response to the amendment filed September 29, 2006. All previous rejections have been withdrawn unless stated below.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action mailed March 23, 2006.

Response to Declaration Under 37 CFR 1.132

The Declaration of Janice M. Troha under 37 CFR 1.132 filed September 29, 2006 is insufficient to overcome the rejection of the instant claims based upon 35 USC 102 and 103 as set forth in the last Office action because: the Declaration shows methods of using the compositions. The claims are directed to a composition, however not a method of use. The intended use of a composition carries no weight in determining patentability because the compositions suggested by the references are substantially the same as the compositions of the instant claims.

Claims

Claim Rejections - 35 USC § 103 (Previous Rejection)

Claims 15, 22-23 and 136-141 were rejected under 35 U.S.C. 103(a) as being unpatentable over Krezanoski (US 4,188,373) in view of Boggs (US 5,358,705). The rejection is maintained in regards to claims 15, 22, 136-137 and 140.

Applicant argues Krezanoski does not disclose N-acetylcysteine (NAC), for any purpose. Applicant further argues based on the Troha Declaration, the disclosure of Boggs et al. would not lead to an expectation that NAC would be efficacious for treatment of mucositis occurring as a side effect of cancer therapy, the pathogenesis of which does not appear to be due to the presence of bacteria.

In response to applicant's argument that Krezanoski does not disclose NAC for any purpose and the disclosure of Boggs et al. would not lead to an expectation that NAC would be efficacious for treatment of mucositis occurring as a side effect of cancer therapy, the test for obviousness is not whether the features of a secondary reference may be bodily incorporated into the structure of the primary reference; nor is it that the claimed invention must be expressly suggested in any one or all of the references. Rather, the test is what the combined teachings of the references would have suggested to those of ordinary skill in the art. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981).

Claim Rejections - 35 USC § 103 – Obviousness (New Rejection)

Claims 1, 15, 19, 31, 35, 38, 133-137, 142-143 and 146 are rejected under 35 U.S.C. 103(a) as being unpatentable over Dobrozsi et al. (US 6,503,955).

Dobrozsi et al. disclose pourable liquid vehicles comprising an aqueous or nonaqueous polymer solution. The vehicles comprise a polyoxyalkylene block copolymer, water and glycols. The copolymer comprises polyoxypropylene and polyoxyethylene and makes up 25% to 77% by weight of the vehicle. Water makes up

5% to 45% of the composition (col. 7, lines 5-25). The glycols used, such as polyethylene glycol, which encompasses claim 31, make up 0 to 70% (col. 6, lines 50-53). Pluronic F-127 is a preferable block copolymer used in the compositions. The pourable liquid vehicle of the disclosed invention were formulated so that the contacting and mixing said vehicles to a mucosal surface of the body, or with some other fluid in the body, triggers the conversion of the pourable liquid vehicle to a more viscous gel-like mixture (col. 4, lines 33-48). The viscosities of the formulated vehicles were measured at room temperature and 37°C the temperature inside the human body. It was disclosed the viscosity of the compositions increased at the higher temperature, therefore encompassing claim 1. The disclosed liquid compositions have a viscosity of less than about 7 pascal seconds, preferably less than about 2 pascal seconds, more preferably less than about 1 pascal seconds (col. 5, lines 12-17), which encompasses no larger than 60cP of the instant claims. The desired value of a composition's triggered viscosity ratio is least about 1.3, preferably at least about 2, more preferably at least about 5, and most preferably at least about 10. The triggered viscosity is defined as the viscosity of the gel divided by the viscosity of the liquid. Using this calculation the gel viscosity is greater than 80cP, which encompasses the instant claims. The pourable liquid vehicles have a number of utilities including delivery of therapeutic agents. These include agents selected from the group consisting of expectorants/mucolytics, antioxidants and mixtures thereof (col. 7 lines 38-51). Expectorants/mucolytics include N-acetylcysteine. The active agents are added to the vehicles ranging up to 5% weight of the total composition according to the disclosed examples, which encompasses claim

15. The reference discloses several different dosage forms including gels, rinses, sprays and liquid filled capsules for intra-oral administration. Flavors and preservatives are also used in the disclosed compositions (see examples), as recited in claims 35 and 38.

The reference differs from the instant claims insofar as it does not disclose specifically using N-acetylcysteine in a composition comprising poloxamers 407. The reference is not anticipatory insofar as one must "pick and choose" from different lists of active agents and poloxamers. That being said, it would have been obvious in a self-evident manner to have selected N-acetylcysteine from one list and poloxamers 407 from another, motivated by the unambiguous disclosure of each individually, and consistent with the basic principle of patent prosecution that a reference should be considered as expansively as is reasonable in determining the full scope of the contents within its four corners.

2) Claims 17, 20, 24-25 and 137, 140 144-145 and 147-148 are rejected under 35 U.S.C. 103(a) as being unpatentable over Dubrozsi et al. (US 6,503,955) in view of Stratton et al. (US 5,861,174).

The primary reference is discussed in subsection 1, above. The reference differs from the instant claims insofar as it does not disclose the compositions comprise 0.1 to 20% of the preferred block copolymer, the compositions comprise about 10% or N-acetylcysteine and the composition were made when the liquid carrier was 5°C.

Stratton et al. disclose pharmaceutical compositions for the delivery of pharmacologically active proteins. The polypeptides make up 0.5% or greater of the disclosed compositions (col. 3, lines 38-45). In one embodiment of the invention, the polypeptide comprises 0.5 to 50% by weight of the compositions (col. 6, lines 46-50). The polymers of disclosed invention provide a sustained release delivery system for active agents or drugs (col. 1, lines 51-53) The delivery vehicle comprises block copolymers, polyoxyethylene-polyoxypropylene namely Pluronic polyols, or poloxamers. Poloxamers have the ability to gel as a function of temperature and polymer concentration. Poloxamers having molecular weights below 10,000, do not form gels at any concentration, therefore Pluronic F-127 and Poloxamer 407 are the polymers of choice for the disclosed invention (col. 2, lines 18-60). These polymers have the characteristics of being liquid at temperatures below room temperature but will form a gel as they are warmed (col. 4, lines 38-41). The aqueous polymer solutions may be formed in two ways, by a cold process or by a hot process. The cold process involves dissolving the polymer at a temperature from about 5°C to 10°C (col. 5, lines 20-34). When adding the polypeptide, it is preferred to add the agent at a temperature of about 0°C to 10°C. These conditions encompass claims 24-25. Raising the sample temperature above the gel point of the poloxamer results in an even distribution of protein particles throughout the polymer gel (col 6, lines 1-7). The copolymer will not form a gel at a concentration outside the range of about 20% to 30% by weight (which overlaps the concentration of the instant claims . but it was discovered other compounds could be added to the compositions in order for the copolymer to form a gel

at concentrations lower than 20% by weight, which encompasses claim 137 as well as claim 20.

The reference differs from the instant claims insofar as it does not disclose compositions comprising glutathione or its precursors and the viscosities of the compositions before and after the temperature change.

It would have been obvious to adjust the amount of poloxamer in the compositions of the primary reference motivated by the desire to obtain the desired characteristics of the composition, such as the removal of the reverse-thermal gelation property as recited in claim 20, as disclosed by the secondary reference.

It would also have been obvious to one of ordinary skill in the art to have used the delivery system comprising 20 to 30 percent poloxamer and theory to deliver the active agents of the primary reference motivated by the desire to provide a sustained release composition that exist in a liquid form and gels when introduced into the body wherein the therapeutic composition is released over a period of time, as disclosed by the secondary reference.

Normally, changes in result effective variables are not patentable where the difference involved is one of degree, not of kind experimentation to find workable conditions generally involves the application of no more than routine skill in the art. In re Aller 105 USPQ 233, 235 (CCPA 1955). It would also have been obvious to one of ordinary skill in the art to have adjusted the amount of N-acetylcysteine in the compositions of the primary reference motivated by the desire to deliver an effective amount of active agent to obtain optimal results as supported by cited precedent.

3) Claims 1, 15, 20, 22, 24-25, 35, 38, 137, 140 and 142-148 are rejected under 35 U.S.C. 103(a) as being unpatentable over Boggs (US 5,358,705) in view of Stratton et al. (US 5,861,174).

Boggs et al. disclose oral compositions for preventing conditions of the oral cavity. The active ingredients in the compositions include N-acetylcysteine complexes, which make up 0.05 to 10% of the compositions as recited in the instant claims. These concentrations are considered "safe and effective", which is defined as an amount of compound or composition sufficient to induce a significant positive modification in the condition being treated, but low enough to avoid serious side effects (col. 4, lines 11-32). The compositions also include surfactants such as Pluronic F-127 and make up 0 to 10% of the compositions. The reference differs from the instant claims insofar as it does not specifically disclose the compositions exhibit thermal-reversible behavior.

The secondary reference is discussed above and disclosed the thermal properties of polyoxyethylene and polyoxypropylene copolymers. It is used as a general teaching to show the surfactants used in the compositions of the primary reference are thermal responsive polymers and do not display thermal responsive gelation at the disclosed concentrations. The reference differs from the instant claims insofar as it does not disclose comprising N-acetylcysteine in the compositions.

It would have been obvious to one of ordinary skill it the art to have used the amounts of poloxamer used in the compositions of the primary reference motivated by the desire to inhibit gel formation but still has an increased viscosity when introduced

into the body to prolong the release of the active agent, as disclosed by the secondary reference.

Claims 1, 15, 17, 19-20, 22, 24-25, 31, 35, 38, 133-137 and 142-148 are rejected.

No claims allowed.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lezah W. Roberts whose telephone number is 571-272-1071. The examiner can normally be reached on 8:30 - 5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin H. Marschel can be reached on 571-272-0718. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Lezah Roberts
Patent Examiner
Art Unit 1614

Frederick Krass
Primary Examiner
Art Unit 1614

APPENDIX C

TO

DECLARATION

OF

GARY J. ROSENTHAL

Copies of:

Krezanoski (US Patent No. 4,188,373)
Boggs et al. (US Patent No. 5,358,705)
Stratton et al. (US Patent No. 5,861,174)

APPENDIX D

TO

DECLARATION

OF

GARY J. ROSENTHAL

Copy of Claims Considered

1. A therapeutic composition useful for treatment of oral mucositis as a side effect of cancer therapy, the composition comprising:

N-acetylcysteine in an amount effective as formulated in the composition to provide therapeutic effect for treatment of the mucositis;

from 5 weight percent to 20 weight percent poloxamer 407;

a carrier liquid comprising water in an amount sufficient as formulated in the composition to interact with the poloxamer 407 to impart reverse-thermal viscosity behavior to the therapeutic composition, wherein the composition exhibits the reverse-thermal viscosity behavior over at least some range of temperatures between 1°C and 37°C;

wherein, at some temperature in a range of from 2°C to 8°C the therapeutic composition is in the form of an aqueous solution with the poloxamer 407 and the N-acetylcysteine dissolved in the water.

15. The therapeutic composition of Claim 1, wherein the N-acetylcysteine comprises from about 0.001 percent by weight to about 50 percent by weight of the composition.

17. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits the reverse-thermal viscosity behavior over at least some range of temperatures between 1°C to 20°C.

19. The therapeutic composition of Claim 1, wherein the biocompatible polymer, as formulated in the therapeutic composition, imparts a reverse-thermal gelation property to the composition with the composition having a reverse-thermal liquid-gel transition temperature within a range of from 1°C to 37°C, so that the therapeutic composition gels as the temperature of the therapeutic composition is increased from below to above the reverse-thermal gel transition temperature.

20. (Previously Presented) The therapeutic composition of Claim 1, wherein the amount of the water, as formulated in the composition, does not interact with the poloxamer 407 to impart reverse-thermal gelation properties to the composition.

22. The therapeutic composition of Claim 1, wherein the poloxamer 407 comprises from 5 weight percent to 20 weight percent of the composition.

24. The therapeutic composition of Claim 1, wherein the poloxamer 407 is dissolved in the water when the composition is at a temperature of 5°C.

25. The therapeutic composition of Claim 24, wherein the N-acetylcysteine is dissolved in the water when the composition is at a temperature of 5°C.

31. The therapeutic composition of Claim 1, comprising a bioadhesive agent that is different than the N-acetylcysteine and the poloxamer 407.

35. The therapeutic composition of Claim 1, comprising at least one taste masking component.

38. The therapeutic composition of Claim 1, comprising at least one preservative component.

133. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits an increase in viscosity from no larger than about 60cP to at least about 70cP when a temperature of the composition is increased from 1°C to 37°C.

134. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits an increase in viscosity from no larger than about 60cP to at least about 80cP when a temperature of the composition is increased from 1°C to 37°C.

135. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits an increase in viscosity from no larger than about 50cP to at least about 70cP when a temperature of the composition is increased from 1°C to 37°C.

136. The therapeutic composition of Claim 1, wherein the composition comprises reverse-thermal gelation properties with a reverse-thermal liquid-gel transition temperature within the range of temperatures.

137. The therapeutic composition of Claim 1, wherein the therapeutic composition comprises from 0.1 to 20 weight percent of the N-acetylcysteine.

140. The method of Claim 137, wherein the therapeutic composition comprises about 10 weight percent of the N-acetylcysteine.

142. The therapeutic composition of Claim 1, wherein:

the therapeutic composition is adapted for delivery to a patient when the therapeutic composition is at a refrigerated temperature in a range of from 1°C to 10°C; and

when the therapeutic composition is at the refrigerated temperature, it is in the form of a flowable medium with each of the N-acetylcysteine and the poloxamer 407 dissolved in the water.

143. The therapeutic composition of Claim 142, comprising from 0.1 weight percent to 25 weight percent of the N-acetylcysteine.

145. The therapeutic composition of Claim 143, comprising from 10 weight percent to 20 weight percent of the poloxamer 407.

146. The therapeutic composition of Claim 143, comprising up to 10 weight percent of the N-acetylcysteine.

147. The therapeutic composition of Claim 143, comprising about 10 weight percent of the N-acetylcysteine.

148. The therapeutic composition of Claim 147, comprising from 10 weight percent to 20 weight percent of the poloxamer 407.

149 The therapeutic composition of Claim 143, wherein when the therapeutic composition is at a temperature of 2°C the therapeutic composition has sufficient fluidity for use as a mouthwash that can be swished in the oral cavity.

150. The therapeutic composition of Claim 143, wherein when the therapeutic composition is at a temperature of 2°C the viscosity of the therapeutic composition is no larger than 60 cP.

151. The therapeutic composition of Claim 143, wherein the carrier liquid is water.

152. The therapeutic composition of Claim 143, wherein the carrier liquid comprises, in addition to the water, at least one component selected from the group consisting of ethanol and a polyol.

APPENDIX E

TO

DECLARATION

OF

GARY J. ROSENTHAL

Copies of:

Liao, YH et al. Biological Monitoring of Exposures to Aluminum, Gallium, Indium Arsenic and Antimony in Optoelectronic Industry Workers. JOEM. 2004. Vol 46. pp 931-936.

Blair, HC et al. Reversible Inhibition of Osteoclastic Activity by Bone-Bound Gallium (III). J Cell Biochem. 1992. Vol 48. pp 401-410.

Alfrey, AC et al. The Dialysis Encephalopathy Syndrome: Possible Aluminum Intoxication. NEJM. 1976. Vol 294. pp 184-188.

Campbell, A et al. Differential Toxicity of Aluminum Salts in Human Cell Lines of Neural Origin: Implications for Neurodegeneration. NeuroToxicology. 2001. Vol 22. pp 63-71.

Biological Monitoring of Exposures to Aluminium, Gallium, Indium, Arsenic, and Antimony in Optoelectronic Industry Workers

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The main objective of this study was to investigate aluminum, gallium, indium, arsenic, and antimony exposures on blood and urine levels in the optoelectronic workers. One hundred seventy subjects were enrolled in this cohort study. Whole blood and urine levels were determined by inductively coupled plasma-mass spectrometry. Blood indium and urine gallium and arsenic levels in the 103 workers were significantly higher than that in 67 controls during the follow-up period. In regression models, the significant risk factors of exposure were job title, preventive equipment, Quetelet's index, sex, and education level. The findings of this study suggest that gallium, indium, and arsenic exposure levels may affect their respective levels in blood and urine. The use of clean, preventive equipment is recommended when prioritizing the administration of safety and hygiene in optoelectronics industries. (J Occup Environ Med. *2004;46:931–936*)

Production of optoelectronic devices consists of four basic steps: (1) production of the IIIA-VA and IIA-VIB metals wafer, (2) design of the integrated circuit, (3) manufacture of the integrated circuit or "chip" on the wafer, and (4) assembly and checking of electronic devices using the finished chip. The final products have been widely used in electronic devices such as microwave devices, laser, light-emitting diodes, photoelectric chemical cells, and semiconductor devices[1,2]

Optoelectronic manufacturing is a metallic-intensive modern industry where workers are potentially exposed to toxic metals of IIA, IIIA, VA, and VIB families, including aluminum (Al), gallium (Ga), indium (In), arsenic (As), antimony (Sb), zinc (Zn), and selenium (Se). These metals or metalloids were known to alter several cellular functions, resulting in eukaryotic cell death and carcinogenesis in animals and clinics studies.[3–13]

Taiwan produces the largest number of CD-Rs and CD-ROMs in the world, accounting for 74% and 46.6%, respectively. The optoelectronic production in Taiwan has increased by 34% from 1993 to 1999; consequently, the number of optoelectronic companies has increased rapidly. Because workers in the optoelectronic companies are potentially exposed to a variety of heavy metals, in this study we examined several heavy metals, including Al, Ga, In, As, and Sb levels, in the blood and urine of optoelectronic workers.

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Materials and Methods

Study Subjects

Workers from three optoelectronic companies, which were established in northern Taiwan in 1991 to 1994, were classified into four exposure categories on the basis of their exposure operation performed, chemicals used, and reference[14]: 1) 15 fabrication equipment preventative maintenance (PM) workers; 2) 52 dopants and thin film (dope film) workers; 3) 36 fabrication supervisors and engineers (SUP/ENG); and 4) 67 office workers (nonexposed).

Questionnaire Information

A self-administered questionnaire was given to the subjects at day before their annual health check-ups, which were arranged by the company's clinic. Scheduling of check-ups was based on personal identification numbers rather than on exposure levels or job titles. Informed consent was obtained from all subjects. The questionnaire included information on age, height, weight, education level, antacids intake, past history of diseases, cigarette smoking, alcohol consumption, job classifications, job histories, and use of protective equipment. Quetelet's index (kg/m^2) was used to evaluate the degree of obesity. Because antacid drugs may contain Al, the history of intake of antacids also was collected. Information on protective equipment, including facemasks, gloves, and booties shoes, also was collected.

Collection and Analysis of Biomonitoring Specimens

The fist spot of urine specimen in the morning and blood sample from the antecubital vein were obtained from the study subjects during their health check-ups. Subjects were instructed not to eat anything for at least 8 hours prior to blood and urine sampling. Blood and urine specimens were kept at –20°C prior to analysis.[15]

Microwave dissolution technique was used to digest blood samples by a Model-MDS-2000 CEM remote Microwave system (CEM, Matthews, NC). A blood sample (2.5 mL) and 4 mL of the Bi-acid solution (3 mL 65% HNO_3 + 1 mL 30% H_2O_2) were transferred into a 125-mL pressure-resistant polytetrafluoroethylene bottle. The sample was digested by six steps to remove the interfering matrix (S1: 250 W, 2 minutes; S2: 0 W, 2 minutes; S3: 250 W, 6 minutes; S4: 400 W, 5 minutes; S5: 650 W, 5 minutes; S6: 400 W, 3 minutes). Digested solution was evaporated completely to remove excess acid and then diluted with deionized water to 6 mL for metal analysis.[16] A urine sample (5 mL) was diluted with Merk 65% HNO_3 solution and then digested at the condition of 300 W for 4 minutes.[17,18]

Trace concentrations of Al, Ga, In, As, and Sb were determined by an inductively coupled plasma-mass spectrometer autoanalyzer.[19] The detection limits (3 SD of 10 unexposed urban residents) and the precision were shown in Table 1. The measurements below a concentration of detection limits were set at half of the detection limit.

Statistical Analysis

Means and median of metal levels in blood and urine specimens, as well as the duration of employment, age, and Quetelet's index, educational level, cigarette smoking, alcohol consumption, and regular use of protective equipment were examined. History of antacids intake was dichotomized as the intake of antacids more than one tablet per week. Cigarette smoking and alcohol consumption were dichotomized as never and ever users.

The chi-square test was used to compare the demographic differences among the three exposed groups and referents. The *t*-test was used to compare the significant differences of metals in blood and urine (normalized transformation) between the total exposed group and referents. The one-way analysis of variance test and post-hoc statistic were used to compare the significant differences of metal levels in blood and urine among the four groups. The Pearson's correlation between each of measured blood and urine levels was calculated. Stepwise multiple regressions were performed with the exposure category as the major predictor variable after reducing to the model all other variables that were significantly different between the total exposed and unexposed groups. All analyses were performed with the SPSS (SPSS Inc., Chicago, IL) for Windows.

TABLE 1
Quality Controls of the Analyses of Metals by an Inductively Coupled Plasma-Mass Spectrometry (n = 10)

Element	Blood			Urine		
	DL (μg/L)	CV (%)	Range	DL (μg/L)	CV (%)	Range
Al	0.171	29.00	0.79–1.91	4.924	12.00	6.190–9.980
Ga	0.016	32.00	0.14–0.16	0.101	23.00	0.230–0.440
In	0.081	15.00	0.11–0.19	0.005	30.00	0.030–0.070
As	0.970	24.00	2.15–4.46	9.980	17.00	15.20–26.20
Sb	0.142	30.00	1.16–2.57	0.003	33.00	0.120–0.380

DL, detection of limit; CV, coefficient of variance.

Results

Table 2 shows the distribution of the demographic data and potential confounders among the three exposed subgroups and referents. The total exposed group had a higher proportion of subjects with males; smoking habits; and use of masks, gloves, and booties than the referents.

Tables 3 and 4 show the mean trace metal levels in blood and urine among the exposed workers and referents. There were significant differences in blood In and urine Ga and As levels among the four groups. Using the one-way analysis of variance test and post-hoc statistics, significant differences were found between PM workers and the referents in blood In and urine Ga and As levels, between dope film workers and the referents in urine Ga levels, and between SUP/ENG workers and the referents in blood In and urine Ga and As levels. Urine As levels in the PM workers (geometric mean = 39.72 ppb) were significantly higher than those in dope film workers (geometric mean = 18.81 ppb, $P < 0.05$).

Blood Al, Ga, In, and Sb levels were positively and significantly correlated ($P < 0.05$) with urine Al, Ga, In, and Sb levels in the total subjects, respectively. Blood Al, Ga, and Sb levels were positively and significantly correlated ($P < 0.05$) with urine Al, Ga, and Sb levels in the total exposed group. Blood Sb levels were positively and significantly correlated ($P < 0.05$) with urine Sb levels in the PM workers, and blood As levels were positively and significantly correlated ($P < 0.05$) with urine As levels in the SUP/ENG workers. However, only blood Sb levels were positively and significantly correlated ($P < 0.05$) with urine Sb levels in the referents. No associations were found between the blood and urine metals levels in dope film workers (Table 5). Based on the analysis of Sb levels resulting from exposure situation, the PM workers, rather than dope film and SUP/ENG workers, showed the highest coefficient of determination ($r^2 = 0.413$).

In stepwise regression models (Table 6), we first used the referents as an external comparison group to examine blood In, urine Ga, and As levels. The regression coefficient (β) of exposure situation is 0.25 ($P < 0.05$) in blood In levels, 0.25 ($P < 0.05$) in urine Ga, and 0.18 ($P < 0.05$) in urine As levels, and the regression coefficient (β) of Quetelet's index was 0.19 in urine Ga levels. However age, duration of employment, sex, educational levels, cigarette smoking, alcohol consumption,

TABLE 2
Demographic Characteristics of Exposed (n = 103) and Referents (n = 67)

Job Category	Exposure Group			Total	Referents
	PM	Dope Film	SUP/ENG		
Number	15	52	36	103	67
				Median	
Duration of employment (months)	32.0	19.0	29.5	23.3	24.2
Age (yrs)	29.2	28.0	28.5	28.3	29.1
Quetelet's index (kg/m^2)	20.9	22.2	23.0	22.3	23.0
				n (%)	
Sex*					
Male	10 (66.7)[a]	28 (53.8)[a]	25 (69.4)[a]	63 (61.2)	22 (32.8)
Female	5 (33.3)	24 (26.2)	11 (30.6)	40 (38.8)	45 (67.2)
Education level					
< college	10 (66.7)	45 (85.6)	26 (72.2)	81 (78.6)	53 (79.1)
≥ college	5 (33.7)	7 (13.5)	10 (27.8)	22 (21.4)	14 (20.9)
Smoking status*					
No	12 (80.0)	40 (76.9)[a]	31 (81.6)	83 (80.6)	63 (94.0)
Yes	3 (20.0)	12 (23.1)	5 (13.9)	20 (19.4)	4 (6.0)
Alcohol consumption					
No	14 (93.3)	47 (90.4)	29 (80.6)	90 (87.4)	61 (91.0)
Yes	1 (6.7)	5 (9.6)	7 (19.4)	13 (12.6)	6 (9.0)
Regular use of mask*					
No	2 (13.3)[a]	15 (28.8)[a]	14 (38.9)	31 (30.1)	33 (49.3)
Yes	13 (86.7)	37 (71.2)	22 (61.1)	72 (69.9)	34 (50.7)
Regular use of gloves*					
No	1 (6.7)[a]	12 (23.1)[a]	15 (41.7)	28 (27.2)	32 (47.8)
Yes	14 (93.3)	40 (76.9)	21 (58.3)	75 (72.8)	35 (52.2)
Regular use of booties*					
No	3 (20.0)[a]	19 (36.5)	12 (33.3)	34 (33.0)	33 (49.3)
Yes	12 (80.0)	33 (63.5)	24 (66.7)	69 (67.0)	34 (50.7)

PM, preventative maintenance; Dope Film, dopants and thin film; SUP/ENG, supervisors and engineers.
* Chi-square test: exposed vs. referents, df = 1, $P < 0.05$.
[a] Chi-square test: PM, Dope Film or SUP/ENG vs. referents, df = 1, $P < 0.05$.

TABLE 3
Metal Levels (PPB) in Blood Among the Exposed Workers and Referents

	Exposure Group				
	PM	Dope Film	SUP/ENG	Total	Referents
Number	15	52	36	103	67
			Mean ± SD (geometric mean, median)		
Al	5.93 ± 5.90 (4.57, 4.83)	5.19 ± 3.80 (3.75, 3.93)	4.51 ± 3.70 (3.15, 3.30)	5.06 ± 4.11 (3.64, 3.60)	3.74 ± 3.08 (2.35, 3.18)
Ga	0.57 ± 0.18 (0.54, 0.55)	0.53 ± 0.44 (0.40, 0.47)	0.44 ± 0.19 (0.40, 0.44)	0.51 ± 0.34 (0.42, 0.47)	0.48 ± 0.23 (0.37, 0.46)
In*	0.21 ± 0.17[a] (0.18, 0.16)	0.20 ± 0.18 (0.40, 0.47)	0.24 ± 0.17[a] (0.40, 0.47)	0.22 ± 0.17 (0.42, 0.47)	0.14 ± 0.12 (0.11, 0.11)
As	7.22 ± 5.09 (5.48, 5.93)	9.25 ± 7.95 (6.30, 7.43)	8.19 ± 3.70 (7.42, 7.28)	8.58 ± 6.36 (6.54, 7.23)	7.85 ± 6.52 (5.81, 6.78)
Sb	0.80 ± 0.68 (0.50, 0.74)	0.71 ± 0.76 (0.40, 0.40)	0.70 ± 0.71 (0.40, 0.44)	0.72 ± 0.72 (0.50, 0.74)	0.64 ± 0.64 (0.37, 0.41)

* Student *t*-test, $P < 0.05$ (total exposed workers vs. referents, df = 1, all data had been normalized).
[a] One-way ANOVA test, and post-hoc Statistic test, $P < 0.05$ (exposure subgroup vs. referents, df = 1).

and use of protective equipment did not significantly affect the blood In, urine Ga, and As levels in the two comparison groups. We finally used the referents as an internal comparison group. PM workers had blood In and urine Ga and As levels that were higher by 22%, 32%, and 45%, respectively ($P < 0.05$), than those in the referents. The regression coefficient (β) of regular use of masks was −0.81 ($P < 0.05$) in urine As levels and of regular use of gloves was −0.48 ($P < 0.05$) in urine Ga levels; otherwise, the regression coefficient (β) of regular use of booties was 0.57 ($P < 0.05$) in Ga levels and 0.66 ($P < 0.05$) in urine As levels. Job title was most commonly correlated with blood In, urine Ga, and As levels. The use of protective equipment was most commonly correlated with urine Ga and As levels except for blood In levels. Dope film workers had blood In, and urine Ga, levels that were higher by 21%, and 22%, respectively, than those in the referents. Job title was most commonly correlated with blood In, and urine Ga levels. SUP/ENG workers had blood In and urine As levels that were higher by 34%, and 20%, respectively, than those in the referents. The regression coefficient (β) of regular use of booties was 0.24 ($P < 0.05$), sex ratio (female = 1) was 0.34, and education level (<college = 1) was −0.32 in urine Ga levels.

Discussion

This study showed elevated blood In, urine Ga, and As levels in workers exposed to Al, Ga, In, As, and Sb when compared with the referents. A post-hoc analysis showed that PM and SUP/ENG workers had significantly higher blood In, urine Ga, and As levels than the referents. These results suggest that heavy exposure to In, Ga, and As metals may affect human blood and urine levels.

TABLE 4
Metal Levels (PPB) in Urine Among the Exposed Workers and Referents

	Exposure Group				
	PM	Dope Film	SUP/ENG	Total	Referents
Number	15	52	36	103	67
			Mean ± SD (geometric mean, median)		
Al	6.09 ± 4.53 (4.76, 4.67)	6.59 ± 6.96 (4.63, 3.03)	6.73 ± 5.84 (4.93, 3.85)	6.56 ± 6.22 (4.75, 3.17)	6.35 ± 5.67 (4.67, 3.26)
Ga*	0.28 ± 0.30[a] (0.19, 0.20)	0.23 ± 0.22[a] (0.16, 0.17)	0.23 ± 0.18[a] (0.17, 0.22)	0.24 ± 0.22 (0.17, 0.19)	0.15 ± 0.15 (0.11, 0.09)
In	0.02 ± 0.02 (0.008, 0.007)	0.03 ± 0.04 (0.010, 0.008)	0.03 ± 0.04 (0.008, 0.015)	0.02 ± 0.04 (0.009, 0.007)	0.02 ± 0.03 (0.009, 0.007)
As*	47.94 ± 28.08[a] (39.72, 44.00)	26.18 ± 22.36[b] (18.81, 22.09)	34.78 ± 29.90[a] (24.25, 27.32)	32.36 ± 26.87 (22.92, 25.34)	24.18 ± 22.50 (16.55, 17.00)
Sb	0.49 ± 0.64 (0.002, 1.64)	0.33 ± 0.42 (0.002, 1.88)	0.35 ± 0.44 (0.002, 1.64)	0.46 ± 0.22 (0.002, 1.88)	0.30 ± 0.43 (0.002, 1.68)

* Mann-Whitney test, $P < 0.05$ (total exposed workers vs. referents, df = 1).
[a] Mann-Whitney test, $P < 0.05$ (exposure subgroup vs. referents, df = 1).
[b] Mann-Whitney test, $P < 0.05$ (PM vs. Dope Film, df = 1).

TABLE 5
The Pearson's Correlation Coefficient Between Blood Al, Ga, In, As, and Sb, and Urine Al, Ga, In, As, and Sb Levels in Optoelectronic Workers

Group (Numbers)	BAl vs. UAl	BGa vs. UGa	B In vs. Uln	BAs vs. U As	BSb vs. USb
Total (180)	0.196*	0.169*	0.194*	0.113	0.291*
Exposure (103)	0.234*	0.258*	0.171	0.107	0.305*
PM (15)	0.160	0.245	0.073	0.216	0.643*
Dope Film (52)	0.204	0.278	0.258	−0.062	0.153
SUP/ENG (36)	0.311	0.300	0.098	0.351*	0.313
Referents (67)	0.125	0.013	0.239	0.101	0.269*

* Significant difference, $P < 0.05$.
Bal: blood Al, Ual: urine Al. BGa: blood Ga, Uga: urine Ga. Bln: blood In, Uln: urine In.
BAs: blood As, UAs: urine As. BSb: blood Sb, Usb: urine Sb.

PM workers had significantly higher urine As levels than the dope film workers. PM workers, rather than dope film and SUP/ENG workers, showed the highest coefficient of determination ($r^2 = 0.413$) with blood and urine Sb levels correlation. The different characters of exposure suggest that PM workers using optoelectronic manufacturing tools could pose a more significant risk than fabrication operation. Frist[20] estimated that PM activities posed a significant risk on workers exposed to airborne, surface contaminants, or physical hazards, especially when protective shielding is removed for repair or maintenance. This is in agreement with our results. Only 15 PM workers were enrolled in this study, and a larger sample size will be necessary to further elucidate the relationship between heavy metal exposure and blood and urine levels.

There were significant relationships between blood Al, Ga, In, and Sb levels and urine Al, Ga, In, and Sb levels in all 170 subjects, respectively. The relationships between blood Al, Ga, and Sb levels and urine Al, Ga, and Sb levels in the total exposed group were higher than the relationships in referents. These results suggested that biological monitoring for the occupational exposure of metals with blood and urine is suitable in optoelectronic industries. We did not find significant differences in blood Al and urine Al, and blood Ga and urine Ga among the four categories groups; however, blood Al and blood Ga levels were correlated significantly with urine Al and urine Ga levels in total exposed group, respectively. These signs indicated that workers exposed to Al and Ga still exist. There were no consistency of exposed effects in blood In levels versus urine In levels, urine Ga levels versus blood Ga levels, and urine As levels versus blood

TABLE 6
The Relationship of Power-Transformed Blood in Levels and urine Ga, As Levels With Risk Factors Among Optoelectronic Workers in Multiple Linear Regression Models

	blood In		urine Ga		urine As	
Variable Group	β (SE)	*P* Value	β (SE)	*P* Value	β (SE)	*P* Value
E vs. R	0.25 (0.03)	$P < 0.05$	0.25 (0.02)	$P < 0.05$	0.18 (0.06)	$P < 0.05$
Quetelet's index (kg/m^2)			0.19 (0.00)	$P < 0.05$		
PM vs. R	0.22 (0.01)	$P < 0.05$	0.32 (0.01)	$P < 0.05$	0.45 (0.04)	$P < 0.05$
Regular use of mask						
Yes vs. no					−0.81 (0.21)	$P < 0.05$
Regular use of gloves						
Yes vs. no			−0.48 (0.06)	$P < 0.05$		
Regular use of booties						
Yes vs. no			0.57 (0.06)	$P < 0.05$	0.66 (0.21)	$P < 0.05$
Dope Film vs. R	0.21 (0.01)	$P < 0.05$	0.22 (0.05)	$P < 0.05$		
SUP/ENG vs. R	0.34 (0.03)	$P < 0.05$			0.20 (0.08)	$P < 0.05$
Regular use of booties						
Yes vs. no			0.24 (0.03)	$P < 0.05$		
Sex						
Male vs. female			0.34 (0.03)	$P < 0.05$		
Education level						
≥ college vs. < college			−0.32 (0.03)	$P < 0.05$		

E, total exposed group; R, referents; SE, standard error; blood In, detected blood In$^{0.0263}$; urine Ga, detected urine Ga$^{0.279}$; urine As, detected urine As$^{0.424}$.

As levels, perhaps as a result of the long biological half-time (approximately 14 to 70 days) of In and higher rate of excretion and changes in tissue retention of Ga and As exposure.[21–24] We found that blood In, urine Ga, and urine As levels are more sensitive than urine In, blood Ga, and As levels in the exposure assessment study. Hence, we suggested that urine specimens are more specific than blood specimens in the biological monitoring for metals in optoelectronic industry workers.

In stepwise regression studies, job title was most commonly correlated with blood In, urine Ga, and As levels. Socioeconomic status also significantly affected urine Ga levels. Optoelectronic workers exposed to Ga, In, and As were confirmed especially in PM workers. The use of protective equipment was the major predictor of PM and SUP/ENG operation exposure in urine Ga and As levels; the use of masks and gloves were both negative correlations, but the use of booties was a positive one. The highest factor was the use of masks, which affected 81% of PMs versus referents in urine As levels. Quetelt's index and education level also affected the urine Ga levels in the exposure assessments. The higher the Quetelt's index, the higher the urine Ga levels found in the optoelectronic workers. The results of this study indicated that protective equipment played an important role when exposure operation occurred, but if the booties were not clean, it would become another container. These results also implied the uptake of these by the exposed workers, including pulmonary and skin routes. Furthermore, the results of this study demonstrated that the importance of preventive equipment prevention and maintenance is equal to the machines. Additional exercise and training and education courses might help decrease urine Ga levels in the optoelectronic workers because exercise help decrease a worker's body mass index, where heavy metals accumulates, and education courses increase a worker's knowledge of industrial safety and health practices. Because the threshold limit values of blood In and urine Ga and As levels are not well known, more work will be needed to clarify the relationship of metals exposure and health effects.

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Reversible Inhibition of Osteoclastic Activity by Bone-Bound Gallium (III)

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Abstract Gallium(III) is a new therapeutic agent for hypercalcemia. Ga^{3+} reduces osteoclast action, but how it inhibits the cell's physiology is unknown. In vivo, 7–12 μM Ga(III) reduces calcium release from bone, but surprisingly, 10–100 μM Ga^{3+} added to isolated avian osteoclasts did not reduce their degradation of L-(5-^{3}H)-proline bone. ^{3}H-proline labels bone collagen specifically, and collagenolysis is an excellent indicator of bone dissolution because collagen is the least soluble component of bone. Ga(III) > 100 μM inhibited osteoclasts in vitro, but also killed the cells. To resolve this apparent conflict, we measured ^{67}Ga distribution between bone, cells, and media. Gallium binds avidly but slowly to bone fragments. One hundred micrograms of bone clears 60% of 1 μM gallium from 500 μl of tissue culture medium, with steady state at > 24 h. Osteoclasts on bone inhibited gallium binding capacity ~40%, indicating a difference in available binding area and suggesting that osteoclasts protect their substrate from Ga binding. Less gallium binds to bone in serum-containing medium than in phosphate-buffered saline; 30% reduction of the affinity constant suggests that the serum containing medium competes with bone binding. Consequently, the effect of [Ga] on bone degradation was studied using accurately controlled amounts of Ga(III) pre-bound to the bone. Under these conditions, gallium sensitivity of osteoclasts is striking. At 2 days, 100 μg of bone pre-incubated with 1 ml of 1 μM Ga^{3+}, with 10 pmoles Ga^{3+}/μg bone, was degraded at 50% the rate of control bone; over 50 pM Ga^{3+}/μg bone, resorption was essentially zero. In contrast, pre-treatment of bone with [Ga^{3+}] as high as 15 μM had no significant effect on bone resorption rate beyond 3 days, indicating that gallium below ~150 pg/μg bone acts for a limited time and does not permanently damage the cells. We conclude that bone-bound Ga(III) from medium concentrations <15 μM inhibits osteoclasts reversibly, while irreversible toxicity occurs at solution [Ga^{3+}] > 50 μM.

Key words: bone resorption, osteoclast, gallium, hypercalcemia, osteoporosis

The skeleton is both the principal supporting organ and the repository of calcium in the air-breathing vertebrates. Hence, bone is produced by osteoblasts and degraded by osteoclasts throughout life to maintain mechanical strength and to regulate serum calcium activity. However, when bone resorption exceeds formation over a prolonged period, osteoporosis and consequent mechanical failure may result. Moreover, rapid uncontrolled bone resorption causes life-threatening hypercalcemia (Muggia, 1990). Medical science has identified factors contributing to these problems, such as reduced estrogen levels in women and production of hypercalcemic factors, like the PTH-like peptide, by tumors (Strewler and Nissenson, 1990). However, general means to control excess bone resorption have proved elusive.

Gallium is a group IIIa (aluminum period) metal that has shown promise as a therapeutic agent for hypercalcemia of malignancy (Warrell et al., 1984). The detailed effects of Ga(III) on cellular physiology are unknown. Solutions at 0.01% w/v (100 μg/ml) have been shown to inhibit osteoclastic bone pitting ~50% during 6 h incubation in vitro (Hall and Chambers, 1990). In contrast, however, gallium nitrate inhibits tumor growth, bone resorption with Paget's disease, and tumor invasion of the skeleton at concentrations 30–50 times lower; effective steady state $Ga(NO_3)_3$ serum levels are 1.9–3 μg/ml (7–12 μM Ga^{3+}) at the clinical doses used (Foster et al., 1986; Matrovic et al., 1990). Clinical studies have also demonstrated the effectiveness of $Ga(NO_3)_3$ in reducing hypercalcemia of

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malignancy in similar dose ranges (Warrell et al., 1984). Gallium nitrate doses higher than ~50 μg/g body weight are fatal (at substantially lower measured serum concentrations) (Foster et al., 1986).

We previously determined that another class of anti-osteoclastic drugs, bisphosphonates, targets to osteoclasts at low micromolar levels via hydroxyapatite binding (Carano et al., 1990). We hypothesized that Ga^{3+}, which accumulates in the skeleton but is chemically dissimilar to bisphosphonates (Bockman et al., 1990), also targets to osteoclasts via local concentration on bone. However, since these chemicals are otherwise disparate, they may affect osteoclast metabolism differently, raising the possibility of synergistic or alternative means for controlling bone degradation and minimizing toxicity.

We sought to resolve the paradoxical finding of osteoclast resistance to Ga(III) at clinically useful doses via studies of the kinetics, distribution, and cellular effects of this drug over several days in vitro. In the present study, we use $^{67}Ga^{3+}$ to measure the concentration and time dependence of gallium binding to bone, and we study the effects of bone bound and soluble gallium (III) on osteoclast activity and viability as a function of time in culture.

METHODS

Cell Cultures

Osteoclasts were isolated from medullary bone of calcium-starved laying hens, *Gallus domesticus*. Briefly, the birds are placed on a diet without calcium carbonate and dicalcium phosphate (Purina 5070-9); since the birds move grams/day of Ca to make their $CaCO_3$ eggshells, large numbers of osteoclasts are produced in the medullary bone to provide the Ca. These cells, as much as 50% of the cellular mass in the medullary bone, can then be easily isolated by washing the medullary bone through a 100 μm nylon filter; the large and dense osteoclasts are further purified and collected by sedimenting the cell population through 70% serum, which raises the osteoclast content to ~80%. To provide osteoclasts at still higher purity, ~95–98%, they are allowed to attach to 25–50 μm bone fragments, and the bone with essentially only osteoclasts attached separated by sedimentation after 18–36 h of incubation in MEM at 37°C in 5% CO_2. Cells were then plated in 2 cm^2 wells in Eagle's minimal essential medium, α-modification, containing 10% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 5 μg/ml cytosine-β-D-arabinofuranoside (α10-MEM) as described (Blair, et al., 1986). Cultures were maintained at 37°C in humidified air with 5% CO_2.

Resorption Assays

Bone-affinity purified osteoclast cultures with 10^4 cells in 1 ml of medium in 2 cm^2 wells of 24-well tissue culture plates were used. To determine bone resorption, 100 μg of 25–50 μm fragments of devitalized rat bone metabolically labeled with L-[5-^{3}H]-proline was added (Teitelbaum, Stewart, and Kahn, 1979). Bone resorption, relative to time in culture, is thus indicated directly by ^3H recovered in supernatants of 10^4 osteoclasts. Activity of cell-free controls was subtracted, and substrate specific activity was then used to convert counts to μg bone resorbed. Substrate specific activity was determined by total hydrolysis (6 N HCl, 24 h at 110°C) and scintillation counting. Unless specified, bone particles were suspended in PBS and added to the osteoclast cultures without any treatment. All experiments included no-cell controls with identically treated substrates.

Pre-binding was generally performed in PBS with gentle agitation for 48 h at 37°C. Bone (as 25–50 μm fragments) was suspended at 100 μg per ml PBS, with the indicated concentrations of gallium (as $Ga(NO_3)_3$), followed by washing and resuspension in tissue culture medium (α10-MEM). When expressed as bound gallium/μg bone, binding is calculated assuming, for convenience, complete binding under these conditions, except at 0.1 mM Ga^{3+}, where binding is taken from Figure 2C. These differences are much less than the variation of cellular measurments using the bone-bound Ga, and therefore regarded as insignificant for our present purposes: cf Figure 2C, 3, and 4. Pre-binding using tissue-culture medium and PBS gave similar results in preliminary osteoclast inhibition studies. However, steady-state binding was slower in α10-MEM and varied from 20% to 70% efficiency, with standard deviations ~3 times those for PBS measurements in ^{67}Ga binding studies (described below). Thus, accuracy of gallium exposure was improved with PBS as the pre-binding medium. Bone powder for resorption assays was ^3H labeled and pre-bound with unlabeled Ga, whereas bone to be used in measuring Ga distribution was unlabeled (but labelled gallium was of course used for these experiments).

Quantifying Gallium Distribution and Bone Association Constants Using ^{67}Ga

One hundred micrograms of 25–50 μm fragments of devitalized rat bone in 500 μl of PBS, or in α10-MEM (α-MEM with 10% FCS, pH 7.40, described above) with and without 10^4 bone attached osteoclasts, were incubated with indicated concentrations of gallium (as $Ga(NO_3)_3$ with 10^{-12} M ^{67}Ga as a tracer). Incubation was at 37°C in sealed tubes (to prevent alkalinization due to loss of CO_2) and assay tubes were centrifuged (10,000*g*, 1 min) to separate bone and supernatant at indicated times. Bone (pellet) and medium ^{67}Ga were then determined by γ-counting. As this isotope has an ~3 day half-life, all samples were counted at the same time (±1 h) to control for decay.

With these data, surface association constants were determined by applying Langmuir's adsorption equation: $\theta_1 = \alpha\mu/(\nu_1 + \alpha\mu)$ (Langmuir, 1917). For our purposes, θ_1 (binding saturation) is Ga_{bone}/Ga_{max}; μ (concentration) is Ga_{free}; and ν_1 (off rate at saturation; the dissociation constant) is the inverse of $K_{association}$. Gallium activities (α) at the concentrations used, all $< 10^{-4}$ M, are assumed to approach unity, making this term unnecessary. Hence, multiplying the right side of Langmuir's adsorption equation by ν_1^{-1}/ν_1^{-1}, making the indicated substitutions, and multiplying through by Ga_{max}, we obtain:

$$Ga_{bone} = \frac{Ga_{max} \cdot Ga_{free} \cdot K_{association}}{(Ga_{free} \cdot K_{association} + 1).}$$

$K_{association}$ values are calculated from a least squares fit of our data (n = 9 for PBS and n = 12 in α10-MEM) to this modified Langmuir adsorption equation (Fig. 3B). Ga_{max} values were calculated for each concentration tested using the $K_{association}$ values from the least squares fit, and are reported as mean ± SD. Ga_{max} and association constants are given as moles Ga/g bone and M^{-1}, respectively; g bone represents the surface of one gram of bone.

Cell Quantification and Protein Synthesis

Viability of cells was measured in parallel with resorption by counting cells excluding trypan blue. Protein synthesis by cultured cells was assessed using [^{3}H]-leucine incorporation (Blair et al., 1989): Cells, incubated in media including 1 μCi/ml of ^{3}H-leucine for 6 h, were washed twice with PBS, and isotope incorporated into macromolecules was fixed (30 min, 10% trichloroacetic acid and 30 min, 5% trichloroacetic acid). Lipids were removed with ethanol/ether (3:1, v/v) to reduce background, and protein solubilized by digestion in 0.1 M NaOH for scintillation counting.

Statistics

Error ranges are standard deviations, except in Fig. 3B, where errors indicate residuals after least squares fitting. Quadruplicate measurements were performed unless noted. Where ^{67}Ga bone-binding was less than no-bone control due to statistical variation, bound Ga is reported as zero and free Ga as 1.0. Where a difference is concluded, comparisons reject the null hypothesis, at 0.05, by Student's t-test.

RESULTS

Gallium Inhibition of Bone Resorption by Osteoclasts

Addition of gallium to osteoclasts cultured on 20–50 μm bone particles produced no inhibition until $Ga^{3+}_{(Total)}$ was 1 mM (Fig. 1). Furthermore, after 48 h at 1 mM Ga^{3+} the osteoclasts had detached from their substrate, did not exclude trypan blue, and were dead, signifying that the mechanism of this inhibition was cell death. This was inconsistent with clinical studies and studies in vivo, which indicated that much lower concentrations of gallium should effectively inhibit bone resorption and that inhibition at low [Ga^{3+}] does not reduce osteoclast number (Foster et al., 1986; Matrovic et al., 1990; Warrell et al., 1984). Previously we reported that binding to bone is critical to the effectiveness of bisphosphonates on osteoclasts (Carano et al., 1990).

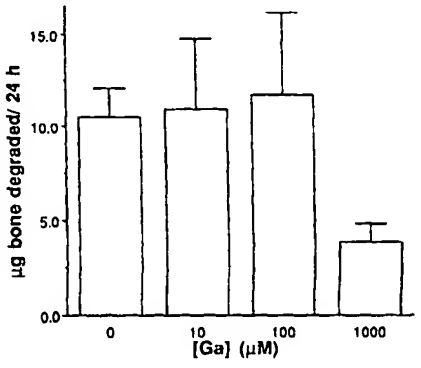

Fig. 1. Effect of gallium on bone resorption by 10^4 osteoclasts in vitro. Bone resorption was measured at 0–48 h of incubation using 100 μg of 25–50 μm fragments of ^{3}H-proline labeled rat bone (Methods). Gallium, as $Ga(NO_3)_3$, was added at time zero.

We reasoned that if gallium was accumulated by bone this would effectively increase its local concentration and inhibit osteoclastic bone resorption without requiring toxic levels of Ga^{3+} in solution. Such a bone matrix accumulation could be quite slow, as the in vivo studies were performed over days to weeks. It was also unknown whether or not osteoclasts would affect Ga^{3+} binding at their matrix attachment, an important corollary measurement if the bone-concentration hypothesis were confirmed. Thus we studied distribution of gallium in media, bone, and cells incubated several days of in vitro.

^{67}Gallium(III) Interaction With Bone Particles

We found that ^{67}Ga(III) binding to bone fragments is avid but slow, with 20–64 h required to reach steady state at initial total ^{67}Ga(III) concentrations of 100 to 0.001 μM (Fig. 2). Significant uptake at 1 nm shows the avidity of bone for the metal. However, at the highest initial concentration, 100 μM, gallium binding by bone reduced medium [Ga] only ~10%, suggesting that binding saturation is significant at micromolar concentrations (see below). Binding to bone particles was qualitatively similar in medium with fetal calf serum added (Fig. 2A) in saline (Figure 2C), and to bone particles with attached osteoclasts (Fig. 2B). In the absence of bone, osteoclasts did not alter ^{67}Ga(III) distribution (not illustrated).

Quantitative analysis, on the other hand, reveals significant concentration and medium dependent differences in gallium-bone binding (Fig. 3). Bone binding, in PBS and α10-MEM with and without cells present, is compared as a function of time, at 1 and 100 μM initial added gallium, in Figure 3A. Uptake was faster in PBS than in serum containing medium. Gallium binding was reduced ~40% in 10% fetal calf serum as compared with saline solution; a further ~50% reduction in ^{67}Ga(III) binding was consistently observed when 10^4 osteoclasts were added to the bone particles 24 h before the addition of gallium. Note that 24-h incubation with osteoclasts has the effect of allowing cells to bind to the bone, covering much of its surface and mea-

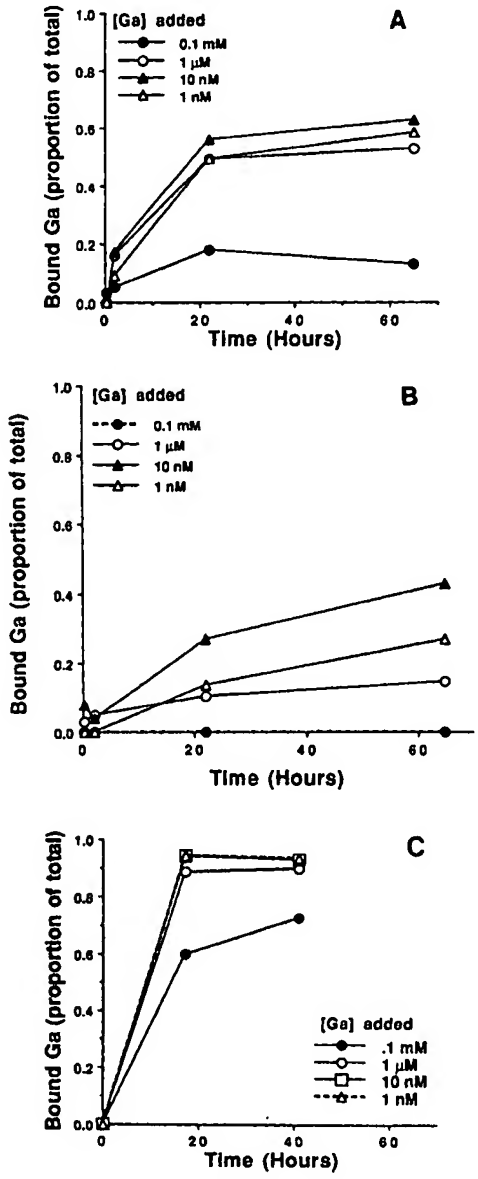

Figure 2.

Fig. 2. ^{67}Ga bound to bone or osteoclast-coated bone from tissue culture medium, or to bone from PBS, as a function of [Ga], 10^{-9}—10^{-4} M, and time. pH of all media was 7.40, and all incubations were at 37.0°C. Standard deviations, not shown for clarity, average 5% of each value (direct comparison of binding in different media is shown, with standard deviations, in Fig. 3A). A: Bound fraction of gallium as a function of time, 0–64 h (free fraction is 1 minus the bound fraction). Tissue culture medium with 10% serum (α10-MEM), 500 μl, pH 7.40, was incubated with the indicated concentration of gallium (III) and 10^{-12} M ^{67}Ga in sealed tubes at 37°C for the indicated times with 100 μg of unlabeled 25–50 μm rat bone. The fraction of bound Ga was then determined by γ-counting after separation of bone from medium by centrifugation. N = 3. B: Bound fraction of Ga as a function of time in α10-MEM, as in (A) above, but using bone aliquots pre-incubated 48 h with 10^4 osteoclasts. Note that the fraction bound (difference from time zero) is ~50% of that resulting when this value was determined in the absence of cells. N = 3. C: Bound fraction of Ga as a function of time, as in (A) above, but with the experiment performed in room air in PBS. Note that the fraction bound is higher than in the tissue culture medium. N = 2.

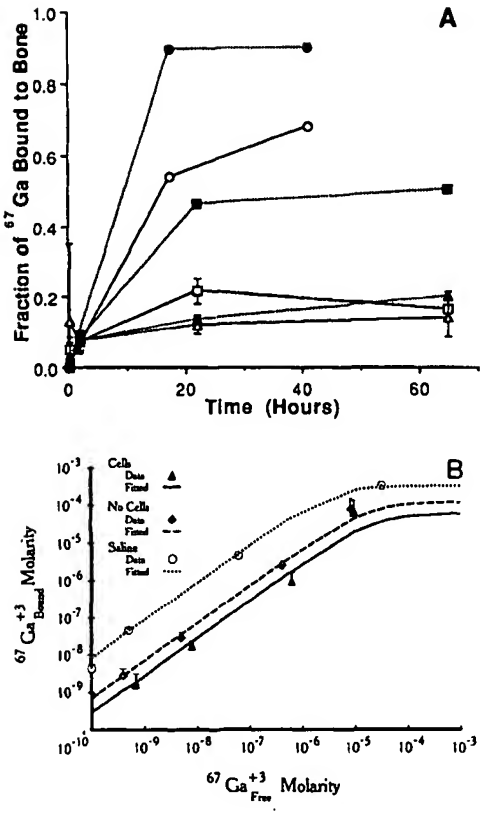

Fig. 3. Comparison of Ga binding time course, and relative fractions bound at steady state, in PBS and serum-containing tissue culture medium (α10-MEM) with and without osteoclasts. A: Comparison of fraction of 100 μM (open symbols and solid lines) and 1 μM Ga (closed symbols and dotted lines) bound to bone as a function of time. Gallium binding to osteoclast-coated (triangles) or unmodified bone (squares) was studied in α10-MEM, and Ga binding to unmodified bone (circles) was also studied in PBS to show by comparison the effect of the serum containing medium. N = 3, mean ± SD, except for PBS (N = 2). Where error is not shown, SD is less than symbol size. B: Relative binding, at steady state, to bone at 10^{-9} to 10^{-4} M initial [Ga]. Binding was assayed and fitted to Langmuir's adsorption equation as described in Methods. Free gallium (i.e., that remaining in solution at steady state, rather than added Ga) is expressed as Log(M); Ga bound is expressed as Log(moles per g bone). The purpose of the log-log scale is to allow comparison over widely differing concentrations. Note that values at $\sim 10^{-4}$ M deviate significantly from linearity, an indication of saturation (except with cells on bone, where Ga_{max} may vary with Ga_{free}, see text).

surably degrading the matrix, while not reducing the amount of bone by more than ~10% (Blair et al., 1986). Thus, the differences in bound Ga^{3+} due to osteoclast attachment are far too large (~50%) to be attributed simply to reduction of available matrix by degradation. This conclusion is strongly confirmed by similar saturation binding of naked and cell bound bone at very high [Ga^{3+}] (Fig. 3B at 10^{-4} M [Ga], discussed below).

Steady-state differences of gallium binding to bone under the various conditions used are expressed as inverse log ratios of bound and free gallium in Figure 3B. Langmuir association constants and gallium adsorption maxima were calculated from these data (Methods and Fig. 3B). Bone-Ga(III) $K_{association}$ in PBS was $79 \pm 3 \times 10^3$ M^{-1}, but decreased ~25% in the presence of serum ($58 \pm 10 \times 10^3\ M^{-1}$) and decreased a further 40% when bone with cells attached was used ($34 \pm 4 \times 10^3\ M^{-1}$). Binding capacity ($Ga_{max}$) was $8.0 \pm 2.6 \times 10^{-4}$ moles/g bone in PBS and decreased in the presence of α10-MEM and α10-MEM + cells to $1.09 \pm 0.16 \times 10^{-4}$ moles/g bone and $0.65 \pm 0.2 \times 10^{-4}$ moles/g bone, respectively (excluding the cell-bone measurement at 10^{-4} M added Ga, which gave $1.01 \pm 0.1 \times 10^{-4}$ moles/g bone, n = 3; see Discussion).

Additionally, under all conditions and concentrations tested, a minimum 10% of the ^{67}Ga is bound from 1 ml of medium onto 100 μg of bone particles (volume < 1 μl), indicating over 100-fold concentration of gallium in the bone fraction at any medium concentration compatible with life (see Discussion). Effective concentration (activity) at the actual bone surface is not directly comparable to solution concentration, but is undoubtedly much higher still: It is established that the interior of mineralized bone matrix is impervious to even such small cations as H^+ (Neuman and Neuman, 1958) and Ga binding to bone is consistent with essentially ideal Langmuir surface adsorption kinetics (Methods and Fig. 3B).

Effect of Bone Associated Ga^{3+}

These results also suggested that osteoclasts would be affected differently by bone pre-incubated with gallium than when gallium was added concurrently with osteoclast exposure to bone. Therefore, we repeated the initial study, but with bone pre-incubated 48 h at 37°C in PBS containing various concentrations of gallium. This substrate was washed prior to osteoclast addition to insure that the experiment would reflect only the effect of bone-bound Ga^{3+}. We found that, under these conditions, gallium was a potent inhibitor of osteoclastic activity. Figure 4 shows inhibition of osteoclastic bone degradation as a function of substrate preincubation [Ga] and time. Half-maximal inhibition was seen

at ~1 μM (yielding 10 pmoles Ga^{3+} per μg of bone, Fig. 3), more than 100 times less than the dose having an effect when added to supernatants (Fig. 1). In various experiments, half maximal inhibition at 48 h incubation was seen following a Ga^{3+}-bone preincubation at 1–3 μM.

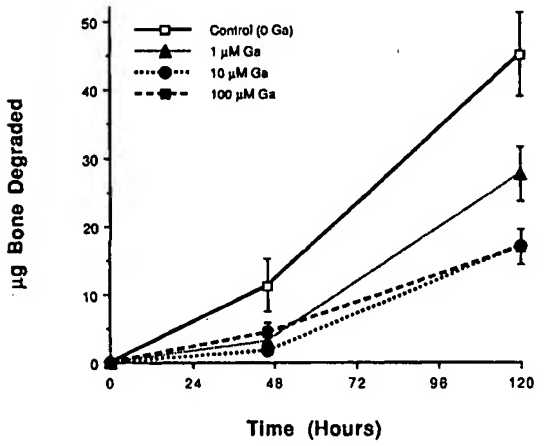

Fig. 4. Time course of bone degradation by 10^4 osteoclasts in α10-MEM, as in Figure 1, when bone had been pre-incubated 48 h at 37°C in PBS in indicated concentrations of gallium nitrate. At 46 and 120 h, supernatants were counted for ^3H activity to determine total bone degradation. Bone-bound gallium was 10 pmoles/μg bone at 1 μM Ga^{3+}, 100 pmoles/μg bone at 10 μM Ga^{3+}, and ~300 pmoles/μg bone at 100 μM Ga^{3+} (Fig. 2C).

Time Dependence of Gallium Inhibition of Bone Resorption

It remained unclear, after the distribution of gallium was studied, why, in the first experiment (Fig. 1), no inhibition was seen at ~10 μM, since by ~48 h even this osteoclast-coated bone should have ~30% as much Ga^{3+} bound as naked bone in PBS (Fig. 2B,C). We had noted, however, that the inhibition of bone resorption by bone associated gallium tended to be less pronounced after longer times in culture (120 h in Fig. 4, for example), suggesting that the effect of bound gallium might decrease with time in culture. Hence, in an attempt to further reconcile resistance to Ga^{3+} in solution and sensitivity to pre-bound Ga^{3+}, we measured the time course of effect of gallium bound to bone. We pretreated bone with 0 to 50 μM Ga(III) (i.e., the only gallium added in these experiments was bone-bound) and determined its osteoclast degradation rate during two time periods (0–72 and 72–135 h). As expected, at 72 h [Ga] over 1.5 mM significantly reduced bone resorption (Fig. 5A). However, at 72–136 h, only bone pretreated with >15 mM Ga^{3+} inhibited bone degradation (Fig. 5B).

Thus, substrate-bound gallium loses its effectiveness after ~48 h (Fig. 4) and is essentially neutralized by 72 h (Fig. 5) under the conditions

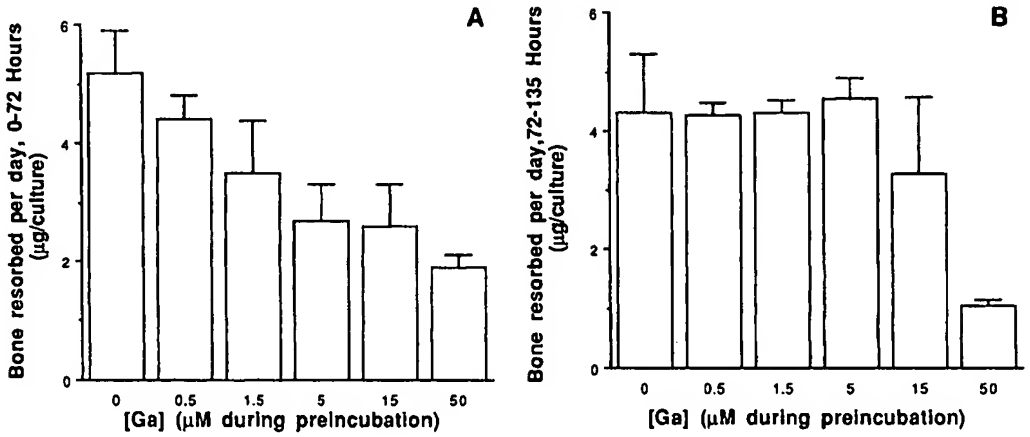

Fig. 5. Rate of bone resorption is inhibited by substrate preincubation in micromolar Ga^{3+} at 0–72 h, but not at 72–135 h of incubation. Bone-bound gallium at time zero, in picomoles Ga^{3+} per μg bone, is 10 times the solution Ga^{3+} concentration, in μM, in which the binding occurred, except at 50 μM Ga^{3+} where saturation would limit bound gallium to <300 pmoles/μg bone (Methods and Fig. 2C). For this experiment, N = 3. **A:** Dose response of bone resorption rate per day when measured from 0–72 h in α10-MEM (as in Fig. 1, but with preincubation of substrate in gallium at the indicated concentrations). Half-maximal inhibition is seen with bone preincubation between 1.5 and 5 μM Ga^{3+}. Note that inhibition is less complete than that measured at 46 h (Fig. 3). This finding is reproducible and indicates that some loss of effectiveness is already likely at 3 days. **B:** Bone resorption rates with substrate pre-incubated in 0–50 μM gallium, as in (A), but measured at 72–135 h of incubation. Only 50 μM gallium gives a significant difference at this time point.

studied (which included a very high osteoclast density and 10% serum, see Discussion). This, and the slow rate at which substrate binding occurs (Fig. 2), would account for the differences of Figure 1 and Figure 4. Put another way, a single dose of Ga appears to lose potency at roughly the same rate that binding occurs. This, incidentally, also suggests that periodic small doses of Ga^{3+} would be advisable for study of gallium effects where pre-binding would be impractical, such as in long-term in vitro incubation.

Effect of Ga(III) on Osteoclast Viability

Dissipation of the gallium effect with time showed that the drug, at least at the lower concentrations, bound to bone and inhibited but did not kill the cells. Indeed, no effect on trypan-blue exclusion was seen at $[Ga^{3+}]$ below 100 μM. This was confirmed by measurment of ^{3}H-leucine uptake by osteoclasts as a function of $[Ga^{3+}]$, which showed no significant effect of the metal on osteoclastic protein synthesis, even at concentrations dramatically depressing bone resorption (Fig. 6).

DISCUSSION

Gallium (III) has been evaluated as an anti-neoplastic drug (Foster et al., 1986). In this setting, however, anti-hypercalcemic properties and effects on bone destruction were prominent (Warrell et al., 1984; Matrovic et al., 1990). Consequently, gallium was proposed as an anti-osteoclastic agent, and indeed Hall and Chambers (1990) found that pit formation by grazing

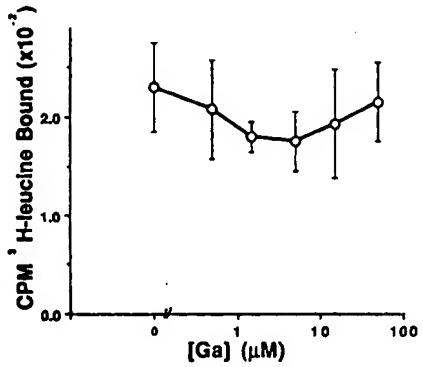

Fig. 6. Six hour ^{3}H-leucine uptake by osteoclasts in α10-MEM after 72 h incubation with bone pre-incubated in 0–50 μM Ga^{3+}. The differences are not significant. This experiment gives variable results at 0.1 mM Ga^{3+}, and does not work at 1 mM gallium, because the cells detach under these conditions (see text).

osteoclasts could be reduced ~50% by 100 μg/ml $Ga(NO_3)_3$ (391 μM Ga^{3+}). However, it is unlikely that this observation represents replication of the clinical activity of gallium in vitro, because the concentrations of $Ga(NO_3)_3$ required to partially inhibit bone resorption would be uniformly fatal in vivo. Indeed, our observations would suggest that, had Hall and Chambers's study been carried to longer time points, $[Ga^{3+}]$ > 100 μM would probably have produced morphological differences between groups (Fig. 1).

Typical serum concentrations for in vivo trials have been 1.9–3 μg/ml (7–10 μM), or ~2% of those used in the cited in vitro study. Hall and Chambers found no morphologic effect of these high concentrations of gallium nitrate at 6 h of incubation. However, we found that concentrations above 50–100 μM Ga(III) are toxic in 48 h of tissue culture. Nevertheless, when we performed an inhibitory dose dependence study in 48-h osteoclast cultures, unreasonably high Ga^{3+} concentrations were, as reported by Hall and Chambers, required to reduce bone degradation (Fig. 1). Therefore, we investigated the possibility that the manner in which osteoclasts are exposed to the drug affects the sensitivity of the cells.

In vivo studies on $^{67}Ga^{3+}$ distribution report that it binds slowly to metaphyseal bone after 14 day exposure to micromolar levels (Bockman et al., 1990), and Hall and Chambers observed that bone exposed 18 h to Ga(III) duplicated the effects of direct application of the drug to tissue culture. Hence, we undertook a detailed quantitative examination of the binding of this metal to bone in vitro using ^{67}Ga(III). To determine whether or not Ga distribution, or time of Ga exposure, affects osteoclast sensitivity to the drug, we also studied the kinetics of Ga(III) action on osteoclastic bone resorption and protein synthesis.

We found that equilibration of Ga(III) distribution in the presence of bone is slow, requiring ~48 h under tissue culture conditions (Fig. 2). Bone-Ga(III) $K_{association}$ in PBS was $79 \pm 3 \times 10^3$ M^{-1}, but $58 \pm 10 \times 10^3$ M^{-1} in the presence of serum, and $34 \pm 4 \times 10^3$ M^{-1} when bone with cells attached was used (Fig. 3A). The decreases in $K_{association}$ with serum containing medium, and further decrease of $K_{association}$ in serum containing medium + cells, indicate reduced affinity of the bone for gallium under these conditions.

Bone-gallium binding capacity (Ga_{max}) was $8.0 \pm 2.6 \times 10^{-4}$ moles/g bone in PBS, and de-

creased in the presence of α10-MEM to $1.09 \pm 0.16 \times 10^{-4}$ moles/g bone. Ga_{max} measurements at $\sim 10^{-4}$ ($\sim$30–80% saturation) to $\sim 10^{-10}$ M gave the same results, confirming the validity of the single-affinity adsorption model for Ga(III) on bone in both of these media. However, the differences of Ga_{max} and $K_{association}$ between serum containing medium and PBS indicate that the serum-containing medium competes with bone for Ga binding, or that medium elements compete for the bone-gallium adsorption sites.

The situation in the presence of cells is more complex. Under these conditions, Ga_{max} was $0.65 \pm 0.2 \times 10^{-4}$ moles/g bone, when calculated using results from 10^{-6}–10^{-10} M free Ga. The effect of the osteoclasts on Ga_{max} at low Ga_{free} suggests that attached cells may reduce the area of high affinity binding (by $\sim$50%). At 10^{-4} M added gallium, Ga_{max} was $1.01 \pm 0.1 \times 10^{-4}$ moles/g bone, more closely resembling the result for the same medium but without cells, although it should be noted that this is the result of triplicate determination only. If confirmed, this result would suggest that osteoclasts affect binding (presumably at the cell attachment site) by reducing affinity, but leave capacity essentially unaffected. Presence of cells did reduce the binding constant, further suggesting that cell-associated bone may have a significantly lower affinity for gallium than naked bone. In the absence of bone, osteoclast cultures did not alter ^{67}Ga(III) distribution, indicating that the cells alone do not bind significant quantities of Ga (at any concentration tested). Thus, the osteoclast may reduce gallium binding by excluding the metal from its bone interface. However, the alternative that the physical environment at the cell's attachment, perhaps its acid pH (Blair et al., 1991), reduces $K_{association}$ would appear to be more likely.

At gallium solution concentrations over $\sim$100 μM, we observed that toxicity and cell death was responsible for inhibition of bone resorption. Gallium concentrations of 100 μM are not effectively bound out of tissue culture media by bone (Fig. 2) and are thus exposed to the cells directly (the higher apparent gallium-binding capacity in PBS is not relevant in this regard). High concentrations of Ga^{3+} in serum may mediate the observed toxicity, at high doses, to other organ systems than bone (Foster et al., 1986).

Because it is easily quantifiable and more efficient than binding from tissue culture medium, we used pre-binding in PBS to determine the effects of bone-bound gallium on osteoclasts. Under these conditions, osteoclasts were exquisitely sensitive to gallium, with half maximal inhibition at 10 pmoles Ga/μg bone, produced by pre-binding 1 μM gallium to bone (Fig. 4). Above 50 pmoles Ga/μg bone, resorption at 48 h was effectively abolished.

We thus propose that the concentration of gallium by binding to the bone permits it to achieve a high local concentration at the site of attachment, which suppresses bone resorption without killing the osteoclast. We had previously proposed that similar bisphosphonate-bone binding is important in the function of the bisphosphonates as anti-osteoclastic drugs, although the specific level of bone binding was not quantified (Carano et al., 1990). Recent observations of Lakatos, et al. (1991), support this proposition. Inhibition of osteoclastic bone resorption by bone-bound gallium occurred at $[Ga^{3+}]$ $\sim$100 times lower than previously reported in vitro. Furthermore, total inhibition was achievable by this method, in contrast to the effect of Ga(III) in solution (Hall and Chambers, 1990). Another remarkable finding differentiating the effects of bone-bound and soluble Ga was that the inhibition by bound Ga is reversible with time, as the bound gallium is released into the medium and diluted or bound to proteins (Fig. 5). Inhibition by bone-bound gallium is not associated with reduced cellular protein synthesis, as indicated by ^{3}H-leucine incorporation into proteins (Fig. 6). These results indicated that the cells had not been killed, and, furthermore, that the biological life of bound gallium has a definite limit, $\sim$48 h at the minimum inhibitory level of bone-bound gallium under the conditions tested in vitro (Figs. 4, 5). This could be an advantage in clinical application of Ga(III), as spontaneous decay of activity with time might prevent complication by secondary hyperparathyroidism and osteitis fibrosa if an excess of drug were to accumulate on bone.

It is important to note that cells in vivo are unlikely to encounter levels of bone-bound Ga^{3+} exceeding $\sim$100 pM Ga^{3+}/μg bone, corresponding to PBS binding from 10 μM solution. This follows from the medium (serum) level required to achieve this level of bone-binding, $\sim$20 μM Ga^{3+}. This level, which is $\sim$twice that required to produce 100 pM Ga/μg bone in PBS (Fig. 2A,C), approximates the serum concentration after a fatal dose, and is hence an approximate physiologic maximum (assuming that bone-

binding from serum is similar to that from serum-containing tissue culture medium). Furthermore, since gallium effects on osteoclasts were reversible below ~100 pmoles/μg bone (Fig. 5), the calculated maximum physiologic bone [Ga] is below the osteoclast toxicity threshold. In other words, our results suggest that under conditions compatible with life, Ga(III) should not reduce the number of functional osteoclasts. Warrell et al. (1984) found no change in bone histology, including number of active osteoclasts, after gallium treatment, supporting this conclusion. In vivo studies, incidentally, used moderate, frequent doses of gallium (Matrovic et al., 1990; Warrell et al., 1984). Our results suggest that this is an ideal way of administering the drug, since the life of the drug is short, and low micromolar [Ga] for a period of days is required to achieve adequate bone binding (Fig. 2). Thus, this approach would likely be more effective while avoiding the toxicity of a large doses.

It is also important to consider that the culture model used differs in other important respects from the situation in the whole organism. Gallium has recently been shown to have significant effects on other bone cells, which are not considered here (Lakatos et al., 1991). Furthermore, the short life of bound gallium observed may be influenced by a culture situation where the osteoclasts cover ~50% of the bone surface. This differs substantially in vivo, except in disease states with greatly increased bone resorption. If, in our culture model, osteoclasts release the surface-bound gallium in attempting to resorb treated bone, Ga would be diluted in the medium and be subject to binding by serum or cellular proteins. It would therefore be less likely to re-bind to the bone surface. Released gallium in solution would not inhibit bone resorption unless its concentration exceeded ~50 μM, which would not be likely to occur, since prebinding of gallium employed < 20 μM $Ga(NO_3)_3$. Therefore, after a cycle of attempted bone resorption it is not surprising that we observe the Ga inhibition diminish. In vivo, where osteoclasts usually cover a smaller fraction of total bone surface, gallium inhibition of bone resorption could in theory be much more persistent, since the osteoclasts would clear a smaller fraction of the total surface per unit time. In the living organism, circulating Ga is cleared fairly rapidly, although significant retention of the metal (as tissue binding) occurs, suggesting that gallium is bound into stable complexes or chemically modified in the presence of cells and becomes inactive (Foster et al., 1986; Warrell et al., 1984). Thus, persistence of the anti-osteoclastic effect in vivo may vary with the rate of bone turnover or with binding to other tissues than bone and may be significantly longer than the ~2 days measured in vitro.

The mechanism of action and time course of Ga(III) effects contrast with bisphosphonate mediated osteoclast inhibition, although matrix binding is important in both classes of compounds (Carano et al., 1990). Bisphosphonates are anti-osteoclastic agents, several of which are metabolic inhibitors at micromolar levels, reducing protein synthesis. Additionally, the anti-osteoclastic effects of bisphosphonates tested in vitro did not dissipate over several days in tissue culture.

The degree of concentration at the site of action of the osteoclast suggests that gallium is a fairly specific inhibitor of this cell at solution activities in the high-nanomolar to low micromolar range. This may be useful both for further studies of osteoclastic activity and clinical applications. However, close attention must be paid to kinetics in reproducing the effect. Under the conditions described here, gallium inhibition is limited to ~48 h after osteoclasts contact Ga(III) carrying bone.

ACKNOWLEDGMENTS

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THE DIALYSIS ENCEPHALOPATHY SYNDROME

Possible Aluminum Intoxication

ALLEN C. ALFREY, M.D., GARY R. LEGENDRE, M.S., AND WILLIAM D. KAEHNY, M.D.

Abstract The aluminum content of muscle, bone and brain was measured in control subjects and in uremic patients on dialysis who had been maintained on phosphate-binding aluminum gels. The mean muscle aluminum was 14.8 ppm, and the trabecular-bone aluminum 98.5 ppm in the patients on dialysis, as compared with 1.2 and 2.4 in control subjects ($P < 0.05$). Brain gray-matter aluminum values in a group of uremic patients on dialysis who died of a neurologic syndrome of unknown cause were 25 ppm as compared with 6.5 ppm in a group of uremic patients on dialysis who died of other causes and 2.2 ppm in control subjects. The fact that brain gray-matter aluminum was higher in all patients with the dialysis-associated encephalopathy syndrome than any of the control subjects or other uremic patients on dialysis suggests that this syndrome may be due to aluminum intoxication. (N Engl J Med 294:184-188, 1976)

DURING the past five to seven years aluminum-containing phosphate-binding gels have been widely used as a method of controlling serum phosphorus levels in uremic patients on dialysis.[1] The orally administered aluminum is assumed to be excreted as an insoluble aluminum phosphate in the feces, with little if any of this element actually absorbed by the patient. However, Clarkson et al.[2] performed balance studies on a group of uremic patients receiving aluminum-containing phosphate-binding gels and found that these patients experienced a positive aluminum balance of 100 to 568 mg per day during the study period. Only a limited number of studies have been performed to determine how the administration of oral aluminum salt alters the body burden of this element in uremic patients. Berlyne et al.[3] reported elevated serum aluminum levels in three of six patients on dialysis receiving oral aluminum salts, whereas, Clarkson et al. found that serum aluminum levels were not consistently affected by aluminum administration in the uremic patients in their study. Parson et al.[4] described elevated bone aluminum levels in a number of patients on chronic hemodialysis who had received aluminum-containing antacids, and Clarkson et al.[2] described slightly increased bone aluminum, but not above the normal range, in two patients in their study. The present study was prompted by the fact that an encephalopathy of unknown cause was seen in a number of patients maintained on chronic hemodialysis in the Denver area. All patients had routinely received aluminum-containing phosphate binding gels for at least two years before the onset of this illness.

A major problem in determining tissue aluminum content has been the lack of a sensitive method for aluminum analysis. In the present study a flameless atomic absorption technic that has a detection limit of 6 ppb for aluminum was used. Bone, muscle and brain aluminum levels were measured in control subjects, uremic patients on and not on dialysis and uremic patients who died of dialysis-associated encephalopathy. In addition tissue aluminum levels were correlated with duration of dialysis, which also reflected the duration of administration of phosphate-binding aluminum gels.

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METHODS

Frontal cortex was obtained from 10 control subjects, nine uremic patients on dialysis and 12 uremic patients with encephalopathy. Bone was obtained from nine control subjects and 20 uremic patients. Muscle was obtained from 13 control subjects, 14 uremic patients and 10 patients with the dialysis-associated encephalopathy. All uremic patients on dialysis had received thrice-weekly dialysis and had been maintained on the equivalent of 90 ml of aluminum hydroxide gel (approximately 2 g of aluminum) daily for the duration of their dialysis treatment. Most of the control subjects had been killed in automobile accidents.

Muscle and bone samples were obtained from the uremic patients by biopsy or at time of autopsy, and brain was obtained at autopsy. The tissue samples were kept in a frozen state until analyzed. The muscle and brain were dried for 16 hours at 130°C. Then dried muscle was ground to a fine powder and extracted four times with 2 ml of ether/petroleum-ether (1/1, vol/vol). The tissue was then redried at 130°C for an hour and reweighed. Bone tissue was obtained from the iliac crest. Adhering tissue and periosteum were removed by scraping with a scalpel. Cortical and trabecular bones were separated, and the marrow removed by washing with a jet of deionized water. After air drying at room temperature the bone tissue was ground in a Wiley mill and passed through a 20-mesh sieve. Partial defatting was accomplished by rinsing four times with petroleum ether (boiling point, 30 to 60°C). The tissue was then reground and passed through a 60-mesh sieve. In brains obtained from patients when sample size was adequate the gray matter was separated from the white matter of the brain with a scalpel after freezing. A 25-mg aliquot of the whole brain as well as gray and white matter was then ashed at 400°C for 16 hours in 10-ml pyrex beakers.

Ten to 20 mg of the dry defatted bone and muscle and the ashed brain were placed in 5 ml of a saturated solution of disodium ethylenediaminetetra-acetate (EDTA) and mixed for two to four hours. Twenty-five microliters of the supernatant was injected with an Eppendorf pipette directly into a graphite furnace for analysis. All glass and plastic containers and plastic pipette tips were pre-rinsed with disodium EDTA to eliminate surface aluminum contamination.

Aluminum determinations were performed with a Perkin Elmer 305B atomic absorption spectrophotometer equipped with an HGA-2100 graphite furnace and deuterium background corrector at a wave length of 309.3 nm. The HGA Controller was modified for a programmable temperature-ramped char cycle by Sienco, Incorporated, Morrison, Colorado.

Because of erratic results and sample contamination found with nitric acid digestion the tissue aluminum was extracted with disodium EDTA. EDTA extraction was used because of the high formation constant between EDTA and aluminum (Log Formation Constant, 16.13).[5] The completeness of the EDTA extrac-

tion of dry defatted tissues was verified by ashing of replicate samples. In addition, analysis of replicate samples of brain was performed after ashing of 400°C in pyrex and 600°C in platinum, with similar results.

The maximum aluminum contamination in tissue preparations was approximately 3 ppb. The detection limit for aluminum with this flameless atomic absorption technic as defined as twice the background was 6 ppb. The sensitivity as defined by 44 milliabsorbance units was 1.1 ppb (27 pg).

Interference with aluminum absorption by other tissue ions was not found. A series of standard addition studies showed that the average recovery of added aluminum to the various types of tissue samples was 99 per cent. The standard curve was linear between background (3 ppb) and 100 ppb, and the standard error of estimate was 3.2 ppb.

Three group comparisons were made with analysis of variance and two group comparisons with the t-test. All values are reported as mean ± S.D. dry defatted weight (bone and muscle) or dry weight (brain).

Results

Tissue Aluminum Content

The mean muscle aluminum concentration was 1.22 ± 0.72 in 13 control subjects, 10.24 ± 19.07 in 14 uremic patients on dialysis and 23.60 ± 18.58 in 10 uremic patients with the dialysis-associated encephalopathy. Both the dialyzed uremic group and the patients with the encephalopathy syndrome had significantly higher aluminum levels than the controls (Table 1). The individual aluminum values are shown in Figure 1.

Bone aluminum levels were measured in nine control bones, bones of three uremic patients not on dialysis and bones of 16 uremic patients on dialysis. There were not enough bone samples from the uremic patients with encephalopathy to evaluate separately, so that they were included in the dialyzed uremic group. The cortical-bone aluminum content was 3.88 ± 1.73 in the control subjects and 46.83 ± 41.16 in the uremic patients on dialysis ($P < 0.0005$). The cortical-bone aluminum content was 8.4 in the uremic group not on dialysis. The trabecular-bone aluminum was 2.39 ± 1.18 in the control subjects and 98.48 ± 60.04 in the dialyzed uremic group on dialysis ($P < 0.0005$). In the uremic patients who were not on dialysis trabecular-bone aluminum was 37.4. Trabecular-bone aluminum was consistently higher than cortical-bone aluminum in the uremic patients (Fig. 2). In addition there was also a good correlation between trabecular-bone and cortical-bone aluminum ($r = 0.93$) (Fig. 3).

The mean mixed brain aluminum content was 1.3 ± 0.68 in six control subjects, 7.63 ± 4.58 in six uremic patients on dialysis and 8.91 ± 4.29 in the 12 patients with encephalopathy. Brain aluminum levels were significant-

Table 1. Muscle Aluminum Values in Controls (C), Uremic Patients on Dialysis (D) and Patients with Encephalopathy on Dialysis (S).

Group	Aluminum Content*
	mg/kg of fat-free solids
C (13)†	1.22±0.72
D (14)	10.34±19.07
S (10)	23.60±18.58
P value:	
C vs D+S	<0.05
C vs D	NS‡
C vs S	<0.01
S vs D	NS

*Mean ± SD.
†Figures in parentheses denote no. of subjects.
‡Not significant.

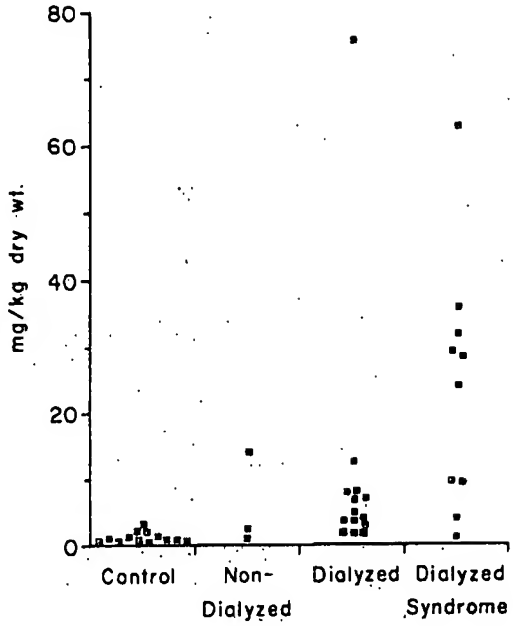

Figure 1. Muscle Aluminum Content in Control Subjects, Uremic Patients Not on Dialysis (Non-Dialyzed), Uremic Patients on Dialysis (Dialyzed) and Uremic Patients with the Encephalopathy Syndrome on Dialysis (Dialyzed Syndrome).

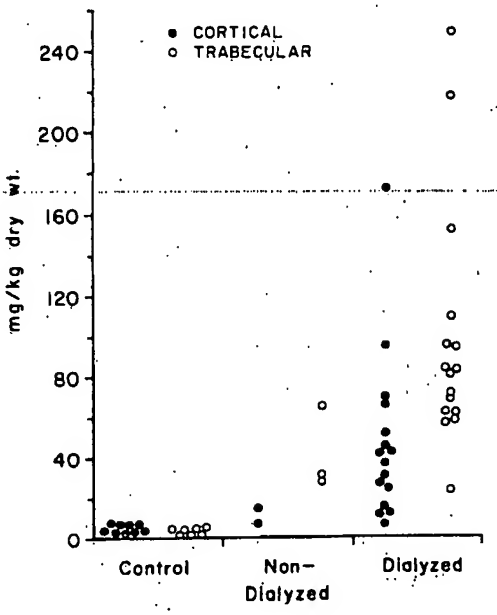

Figure 2. Cortical-Bone and Trabecular-Bone Aluminum Content in Control Subjects, Uremic Patients Not on Dialysis (Non-Dialyzed) and Uremic Patients on Dialysis (Dialyzed).

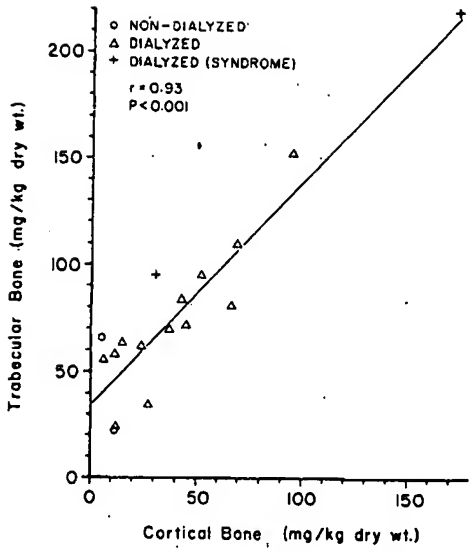

Figure 3. Correlation between Trabecular-Bone Aluminum and Cortical-Bone Aluminum Levels in Uremic Patients.

ly higher in both uremic groups as compared to controls ($P < 0.01$) (Table 2). The mean gray-matter aluminum was 2.18 ± 0.69 in five controls subjects, 6.5 ± 2.93 in seven patients on dialysis, and 24.98 ± 9.1 in six uremic patients with encephalopathy who were on dialysis. The uremic patients with encephalopathy had significantly higher gray-matter aluminum content than either the uremic patients on dialysis ($P < 0.01$) or the control subjects ($P < 0.01$) (Table 2).

The white-matter aluminum content was 2.00 ± 0.63 in five controls, 3.81 ± 1.83 in the uremic group on dialysis and 5.59 ± 1.88 in the patients with encephalopathy (Table 2). In the uremic population with elevated brain aluminum levels the aluminum content in gray matter was consistently higher than that in white matter (Fig. 4).

Correlation between Duration of Dialysis and Tissue Aluminum

There was a significant correlation between muscle aluminum content and duration of dialysis ($r = 0.40$, $P < 0.01$) (Fig. 5). In general, patients with the encephalopathy syndrome tended to have higher muscle aluminum levels at any specific period than patients on dialysis without this syndrome (Fig. 5).

There was also a significant correlation between bone aluminum levels and duration of dialysis ($r = 0.80$, $P < 0.001$) (Fig. 6).

Although there was no correlation between white-matter aluminum content and duration of dialysis there was a significant correlation between gray-matter aluminum content and duration of dialysis ($r = 0.71$, $P < 0.01$). The gray-matter aluminum content in the patients with encephalopathy was greater at all periods than in the other patients on dialysis (Fig. 7).

Table 2. Brain Aluminum Values in Controls (C), Uremic Patients on Dialysis (D) and Patients with Encephalopathy on Dialysis (S).

Group	Aluminum Content*		
	Mixed Brain	White Matter	Gray Matter
	mg/kg of dry solids		
C	1.30±0.68 (6)†	2.00±0.63 (5)	2.18±0.69 (5)
D	7.63±4.58 (6)	3.81±1.83 (7)	6.50±2.93 (7)
S	8.91±4.29 (12)	5.59±1.88 (7)	24.98±9.10 (6)
P value:			
C vs D+S	<0.01	<0.05	<0.01
C vs D	<0.05	NS‡	NS
C vs S	<0.01	<0.01	<0.01
S vs D	NS	NS	<0.01

*Mean ± SD.
†Figures in parentheses denote no. of subjects.
‡Not significant.

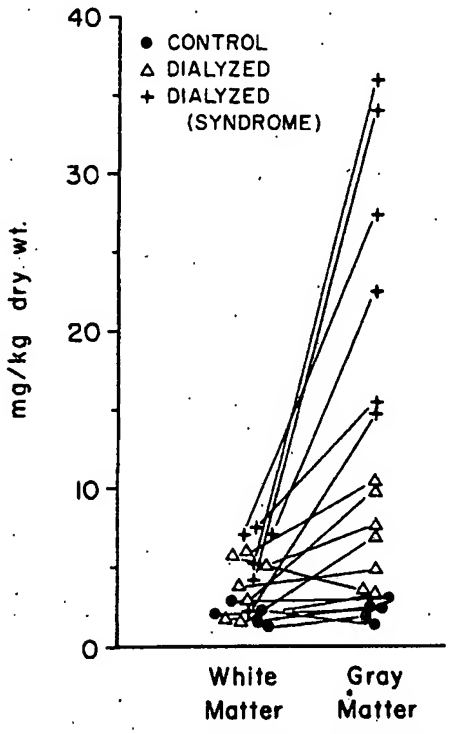

Figure 4. Aluminum Levels in Gray and White Matter of Control Subjects and Uremic Patients with and without the Encephalopathy Syndrome.

Discussion

Flameless atomic absorption technics have been used to measure a variety of different trace elements,[6,7] and for a number of these elements this method has proved to be as sensitive as, or more sensitive than, most other currently available analytical methods. To our knowledge there is only one other publication in which aluminum was measured in biologic samples with flameless atomic absorption technics.[8] In the present study the aluminum content of tissues from control subjects determined by flameless atomic absorption compares quite favorably with tissue values determined by other analytical methods.[9] The flameless atomic-absorption method offers a number of advantages because of its excellent sensitivity for alumi-

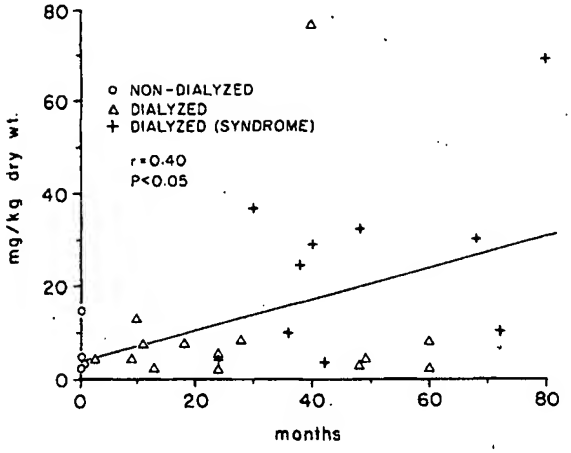

Figure 5. Correlation between Duration of Dialysis (Months) and Muscle Aluminum Content.

num. Very small samples can be used, so that biopsy specimens are usually adequate. Since aluminum is ubiquitous, potential sources of contamination are great. With the described EDTA extraction method and flameless atomic absorption, tissue handling is reduced to a minimum, and most potential sources of external aluminum contamination are avoided.

Tissue burdens of aluminum were found to be markedly altered in uremic patients on dialysis receiving aluminum-containing phosphate-binding gels. In the uremic patients the muscle aluminum content was 10 to 20 times and trabecular-bone aluminum 40 to 50 times control values. Although cortical-bone aluminum was also higher in uremic patients than in controls it was not as greatly increased as trabecular-bone aluminum. These studies suggest that in evaluation of bone aluminum stores, aluminum content of both cortical and trabecular bone should be measured. The total brain aluminum was also found to be significantly higher in uremic patients. However, when aluminum in white and gray matter was measured separately it was found that the major alteration in brain aluminum concentration in uremic patients occurred in the gray matter.

It is unknown whether the body burden of aluminum is also increased in patients with normal renal function who are given aluminum-containing antacids over an extended time, such as patients with chronic peptic-ulcer disease, since this information is not available in the literature. In addition, these studies do not conclusively show that the source of the excess tissue burden of aluminum in uremic patients on dialysis is the aluminum-containing phosphate-binding gels. However, the large amount of aluminum consumed daily by the patients on dialysis in the present study (approximately 2 g) and in that of Clarkson et al.[2] showing a positive aluminum balance in uremic patients receiving comparable dosages of aluminum-containing salts tends to incriminate these compounds. Other sources of aluminum exposure have not been identified. The aluminum content of the water used for the preparation of the dialysate has repeatedly been measured and found to be negligible. We have also been unable to show any net aluminum movement across the dialyzing membrane during the dialysis procedure.

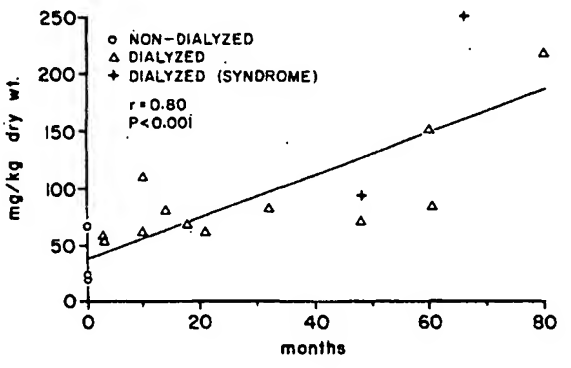

Figure 6. Correlation between Duration of Dialysis (Months) and Trabecular-Bone Aluminum Levels.

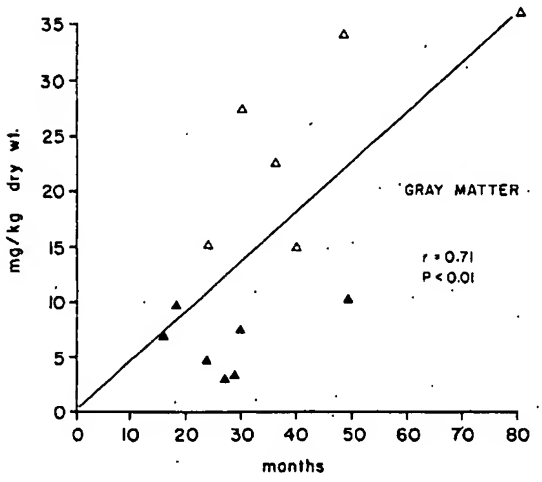

Figure 7. Correlation between Duration of Dialysis (Months) and Aluminum Levels in Brain Gray Matter.
The open triangles denote patients with the dialysis-associated encephalopathy syndrome.

The dialysis encephalopathy syndrome, which has been uniformly fatal, has been described in patients from several different dialysis units.[10-12] The majority of patients in whom this syndrome develops have been on intermittent hemodialysis from three to seven years before the onset of symptoms. In one group of patients maintained at the Denver Veterans Administration hospital this encephalopathy syndrome was the single most common cause of death. Seven of 28 patients treated with intermittent hemodialysis on this program for periods in excess of three years died of this disease whereas only two died of other causes. In addition one of the 19 patients in this group, currently alive, has the dialysis encephalopathy syndrome. The clinical features, which are a mixed dysarthria-apraxia of speech,[13] asterixis, myoclonus, dementia, focal seizures and an abnormal electroencephalogram showing generalized slowing, with multifocal bursts

of delta activity and spikes, have been similar in all patients, suggesting a common origin.[10-12] Complete autopsies have been performed on 12 patients who died of this syndrome. Both gross and microscopical neuropathologic abnormalities have been minimal. Evidence of a slow-virus disease is also lacking in that one patient's brain was injected into primates over 3½ years ago, and to date these animals have experienced no adverse effect. A number of the above features of this neurologic syndrome suggest that it is some type of toxic or metabolic encephalopathy.

It is of interest that brain gray-matter aluminum was higher in all patients with the dialysis-associated encephalopathy syndrome than any of the control subjects or uremic patients on dialysis who died of other causes. With the exception of aluminum other trace-element abnormalities have not been consistently observed in patients who have died of the dialysis encephalopathy syndrome.[10] There is some indirect evidence that this neurologic syndrome may be related to aluminum toxicity. The disease was initially noticed in the Denver dialysis population in 1971, approximately 2½ years after phosphate-binding aluminum had been routinely prescribed to our dialysis population. Since 1971 14 cases of this syndrome have occurred in Denver patients on dialysis. The majority of patients in whom this syndrome has developed have been on dialysis and aluminum-containing salts for over three years. Since brain gray-matter aluminum levels have been shown to vary directly with duration of dialysis the neurotoxicity of aluminum would also be expected to be manifested in patients who had been maintained for extended periods on dialysis. Furthermore, the clinical features of this illness suggest that it is a disease of the gray matter, the area of the nervous system where aluminum accumulates. Finally, one reason that this disease may be seen in some dialysis centers and patients and not in others is that the enthusiasm with which aluminum phosphate-binding gels are prescribed in different dialysis centers and the patients' acceptance of these agents vary considerably.

A number of studies have shown that high concentrations of aluminum can be toxic to the nervous system. In laboratory animals aluminum hydroxide applied directly to the cerebral tissues induces an epileptogenic seizure,[14] and chronic subarachnoid injection of aluminum salts causes a progressive encephalopathy.[15] In addition, Miller and Levine[16] have shown in vitro that neuroblastoma cells cultured in an aluminum-phosphate-containing medium have decreased acetylcholinesterase activity, increased protein content, a higher rate of leucine incorporation and morphologic changes.

McLaughlin et al.[17] described one patient employed as a ball-mill operator in an aluminum flake-powder factory in whom an encephalopathy developed that had many of the features of the dialysis encephalopathy syndrome, including the speech disorder and electroencephalographic abnormalities. Brain aluminum levels were found to be approximately 20 ppm dry weight. In addition Crapper et al.[18] have recently shown that brain aluminum levels are increased in patients with Alzheimer's disease, and have suggested that aluminum toxicity may be involved in the pathogenesis of this disease.

Although the present study does not conclusively show that aluminum intoxication is responsible for the dialysis encephalopathy syndrome, the fact that tissue stores of aluminum are markedly altered in uremic patients on dialysis receiving aluminum salts by mouth suggests that alternative methods for the control of serum phosphorus levels should be sought. It appears that with the use of more efficient dialyzers, phosphate removal during dialysis can be improved. In addition if zero magnesium-containing dialysate was used, magnesium-containing antacids might be safely used to control serum phosphate levels.

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Differential Toxicity of Aluminum Salts in Human Cell Lines of Neural Origin: Implications for Neurodegeneration

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Abstract

Aluminum is highly oxophilic and its minerals are usually found surrounded by six oxygen atoms. A role for the metal has been established in dialysis encephalopathy and Al-induced osteomalacia. The metal has been implicated in Alzheimer's disease but the issue is at present controversial. Human cell lines of neural origin were utilized to study the effect of lipophilic aluminum acetylacetonate and non-lipophilic aluminum sulfate on cell proliferation and viability. Although analysis of Al species in the cell culture media demonstrated that there are positively charged Al species present in solutions prepared with both Al salts, only the aluminum acetylacetonate salt caused changes in cell proliferation and viability. Therefore, the lipophilic nature of the organic Al salt is a critical determinant of toxicity. The effect of aluminum acetylacetonate was dose-dependent and time-dependent. Neuroblastoma (SK-N-SH) cells were more susceptible to decreased cell proliferation although the lipophilic Al salt was more toxic to the glioblastoma (T98G) cells. While the toxicity of aluminum acetylacetonate was inhibited in the T98G cells by the addition of phosphate, the same treatment did not reverse cell death in the SK-N-SH cells. Thus, the mechanism of Al toxicity appears to be different in the two cell lines. It is possible that the principal neurotoxic target of the metal is glial and when these cells are in a compromised state, this may secondarily impact the neuronal population and thus eventually lead to neurodegeneration. © *2001 Elsevier Science Inc. All rights reserved.*

Keywords: Aluminum; Neuroblastoma; Glioblastoma; Cytotoxicity

INTRODUCTION

Approximately 8% of the earth's crust is composed of aluminum by weight. In biological systems, the trivalent cation is rarely present as an ion because it forms extensive complexes with biologically available ligands. Since Al has a high charge to radius ratio, it is predicted that the metal prefers ionic rather than covalent bonding (Berthon, 1996). At neutral pH, the salt undergoes extensive hydrolysis and $Al(OH)_3$ is produced. As the solution ages, $Al(OH)_4^-$ is also present and this leads to precipitation of Al in solutions (Corain et al., 1996).

Fresh human brain tissue, from subjects who were healthy based on autopsy findings and review of medical history, has been collected and analyzed for aluminum content. The brain contains approximately 0.399 ± 0.27 μg/g dry weight of Al in the gray matter and 0.339 ± 0.3 μg/g dry weight of Al in the white matter (Bush et al., 1995). Until recently, it was generally believed that this burden of aluminum was harmless. However, a direct causal role for the metal has been established in dialysis dementia (Alfrey et al., 1976) and microcytic anemia without iron deficiency (Touam et al., 1983). Aluminum has also repeatedly been implicated in the etiology of neurodegenerative disorders although the issue is controversial (Crapper et al., 1973; Good et al., 1992; Bjertness et al., 1996).

The neurotoxicity of aluminum is directly linked to its bioavailability. Since ingestion of Al from both the

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diet and drinking water is the most common form of human exposure, the main route of Al absorption is through the gastrointestinal tract (Exley et al., 1996). Studies in rats estimate that the total aluminum absorbed is approximately 0.1% of the total aluminum ingested (Jouhanneau et al., 1997). In a recent study, two human volunteers were given a single dose of ^{26}Al in tap water. The rate of absorption was found to be 0.22% (Priest et al., 1998). The bioavailability of Al is dependent on the metal's speciation in an aqueous environment and this in turn is dependent on the anion species to which the metal is complexed with (Smith, 1996). We had previously shown that although a 48 h treatment with aluminum sulfate does not change cell viability, it increases oxidative events in glial but not neuronal rodent cell lines. Since Al is thought to be complexed to lipophilic moieties in biological systems, the current study looks at the effect of aluminum acetylacetonate in a human cell line model. It was found that while aluminum sulfate did not have an effect, aluminum acetylacetonate caused a dose-dependent and time-dependent decrease in the cell viability of the two cell lines tested. It was also established that the lipophilic Al salt is more toxic to the glioblastoma (T98G) cells compared to the neuronal SK-N-SH cells, which suggests that glial cells may be the principal targets of Al-induced neurotoxicity.

MATERIALS AND METHODS

Materials

Human glioblastoma (T98G) cells and human neuroblastoma (SK-N-SH) cells were purchased from the American Type Culture Collection (ATCC). Aluminum acetylacetonate was from Aldrich Chemical Company, Inc. (Milwaukee, WI). The Tris buffer salt was from Boehringer Mannheim (Indianapolis, IN). The cell proliferation and cell viability/cytotoxicity kits were from Molecular Probes, Inc. (Eugene, OR). All other chemicals used were from Sigma Co. (St. Louis, MO). All tissue culture supplies were obtained from GIBCO Co. (Grand Island, NY).

Methods

Cell Maintenance and Growth

Human cell lines were grown in minimum essential medium (MEM) with α modification containing 10% fetal bovine serum. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. They were grown in 96 well plates coated with poly-lysine (MW: 70,000–150,000). The aluminum and sodium solutions were prepared and sterile filtered prior to dosing and the salts were added at a final concentration of 0, 2, 10, 100 and 500 μM. After dosing, the media was removed and the cells were rinsed twice with sterile 50 mM Tris–HCl buffer at pH 7.4.

Morin Assay

Aluminum and sodium solutions at 10, 100, and 500 μM were prepared and sterile filtered prior to analysis. The levels of positively charged aluminum species were assayed using the morin dye. Morin, which is a negatively charged compound, can selectively bind aluminum and upon binding to the metal, form a fluorescent complex that can be quantitated by a fluorometer (Browne et al., 1990). For the determination of Al concentration in cells, pellets were washed four times with 50 mM Tris buffer at pH 7.4 to remove any traces of the media. The membranes were dissolved by diluting the cells in 2 ml of Tris buffer containing 1% triton. The morin dye (20 μM) was added to each sample. The samples were incubated for 1 h at room temperature. The response was measured using a FL600 microplate fluorescence reader. The wavelength was set at 420 nm excitation and 515 nm emission.

Cell Proliferation Assay

Cells were gently washed with sterile Tris–HCl buffer at pH 7.4 and then frozen overnight at −70°C. The number of attached cells was determined using the CyQuant cell proliferation assay kit (Molecular Probes, OR). Briefly, cells were thawed at room temperature and treated with 200 μl of the dye diluted in cell lysis buffer. The dye rapidly binds to nucleic acids and exhibits strong fluorescence when bound. The plate was incubated at room temperature for 5 min and the fluorescence was measured. Wavelengths were set at excitation 480 nm and emission 520 nm. A standard of cells counted with the hemacytometer was used to determine the actual cell number.

Cell Viability Assay

The viability/cytotoxicity kit (Molecular Probes, OR) was used to measure cell survival after treatment. This assay is based on the simultaneous measurement of the fluorescence of two dyes. Live cells are determined by the retention of the calcein AM dye, which is non-fluorescent and upon enzymatic conversion by ubiquitous intracellular esterase activity, becomes intensely fluorescent. The EthD-1 dye can only enter

cells with damaged membranes and there it binds to nucleic acids and produces a strong red fluorescence. A final concentration of 1 μM calcein AM and 2 μM EthD-1 was used in the assay.

Since 500 μM of aluminum acetylacetonate completely detached cells form the plate, the cells in the supernatant were collected and analyzed for percent viability. Upon addition of the dye, samples were incubated at room temperature for 45 min. The fluorescence due to calcein was measured at excitation 485 and emission 530 while that of EthD-1 was measured at excitation 530 and emission 645. The percentage of live and dead cells was calculated based on the intensity of fluorescence and a standard of live and dead cells. The dead cells were prepared by treatment with 0.25% digitonin for 10 min. For the effect of phosphate on cell attachment and subsequent viability, only the attached cells were analyzed.

Atomic Absorption Spectroscopy

Cells were grown in 30 mm × 100 mm plates. When the cells reached confluency, they were treated with 100 μM of aluminum acetylacetonate or aluminum sulfate for 48 h. The cells were then washed four times with 5 ml of Tris–HCl-7% NaCl (pH 7.4). After the last wash, buffer was added and the cells were gently scraped off the plate. The samples were then centrifuged for 4 min at 1000 *g* and the supernatant was removed. The resulting pellets were frozen overnight at −70°C. The samples were then shipped on dry ice to Desert Analytics (Tucson, AZ) for evaluation of aluminum content by atomic absorption spectroscopy.

Statistical Analysis

The difference among groups was assessed using one-way Analysis of Variance followed by the Student's *t*-test.

RESULTS

Aluminum Speciation in Cell Culture Media

Extensive hydroxylation of Al in solution has lead researchers to believe that at physiologic pH, the metal is scarcely soluble and has formed aggregates (Corain et al., 1996). The level of positively charged Al complexes was determined in fresh medium (Table 1) and also in medium that was incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 (Table 2). The assay demonstrated that there was a dose-dependent, nonlinear change in the amount of positively charged Al species in the medium. When the morin dye was added to 500 μM of aluminum sulfate, the increase in fluorescence was greater than when the dye was added to 500 μM of aluminum acetylacetonate. This may be because the latter compound is more metastable and does not dissociate and speciate as easily as aluminum sulfate. When morin was added to the sodium salts

Table 1
Al-morin complex formation in cell incubation medium[a]

Sample	Al concentration (μM)	Morin-Al complex formation (arbitrary units of fluorescence); value ± S.E.
Medium alone	0	−0.04 ± 0.26
Medium + morin	0	6.88 ± 0.45
Medium + aluminum acetylacetonate	10	7.63 ± 0.57
	100	16.12 ± 2.30*
	500	35.98 ± 5.12*
Medium + sodium acetylacetonate	10	6.33 ± 1.10
	100	5.62 ± 0.61
	500	5.63 ± 0.86
Medium + aluminum sulfate	10	7.68 ± 0.68
	100	15.90 ± 1.98*
	500	51.32 ± 5.76*
Medium + sodium sulfate	10	6.63 ± 0.93
	100	4.68 ± 0.76
	500	5.90 ± 0.78

[a] Values are mean ± S.E. (n = 4–5). Each sample was assayed in duplicates on separate days. An amount of 20 μM morin was present in all Al-containing medium.

Table 2
Al-morin complex formation in cell incubation medium after a 48 h incubation at 37°C in a humidified atmosphere of 5% CO_2[a]

Sample	Al concentration (μM)	Morin-Al complex formation (arbitrary units of fluorescence); value ± S.E.
Medium alone	0	−0.06 ± 0.08
Medium + morin	0	2.93 ± 0.56
Medium + aluminum acetylacetonate	10	8.35 ± 1.09
	100	18.42 ± 1.65*
	500	22.53 ± 2.82*
Medium + sodium acetylacetonate	10	3.73 ± 0.11
	100	3.42 ± 0.44
	500	2.60 ± 0.90
Medium + aluminum sulfate	10	11.30 ± 0.98*
	100	14.90 ± 1.34*
	500	26.83 ± 4.56*
Medium + sodium sulfate	10	3.50 ± 0.55
	100	3.84 ± 0.43
	500	4.48 ± 0.56

[a] Values are mean ± S.E. (n = 3–5). Each sample was assayed in duplicates on separate days. An amount of 20 μM morin was present in all Al-containing medium.
* Value is significantly different ($P < 0.01$) than the corresponding value using the morin dye in cell culture medium.

fluorescence was comparable to the control values (Table 1).

After a 48 h incubation at 37°C in a humidified atmosphere of 5% CO_2, the media composition of the 500 μM Al solutions changed in such a way that the fluorescence of the Al-morin complex decreased compared to the values obtained with freshly prepared Al-containing solutions (Tables 1 and 2). This effect was not apparent when lower concentrations of aluminum were used. The formation of polymeric and colloidal Al complexes may be responsible for the decrease in the amount of the positively charged Al species. Again, the level of fluorescence did not change significantly upon addition of sodium sulfate or sodium acetylacetonate (Table 2). The non-linear changes seen were probably due to the formation of colloidal Al species, which occur as the solution ages (Corain et al., 1996).

Effect of Aluminum on Cell Proliferation

Aluminum sulfate, at concentrations of 2–500 μM, did not significantly affect the rate of cell proliferation or cell attachment in either the neuroblastoma or the glioblastoma cells (Figs. 1 and 2). On the other hand, aluminum acetylacetonate detached cells and decreased the rate of cell growth, both dose-dependently and time-dependently (Figs. 3 and 4). As early as 4 h after exposure to 500 μM Aluminum acetylacetonate, the cells begin to separate from the growth

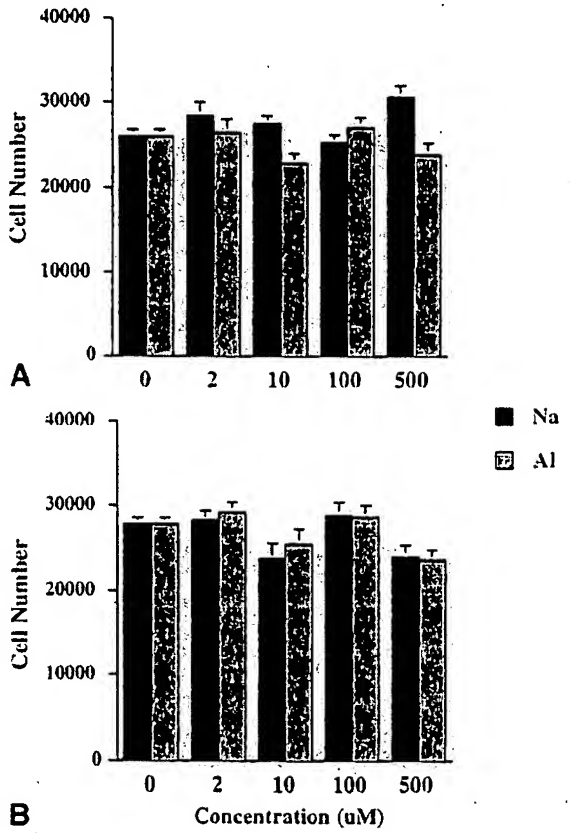

Fig. 1. Cell proliferation in human SK-N-SH cells treated with different concentrations of aluminum and sodium sulfate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean ± S.E. (n = 12). Two to three experiments were conducted on separate days with freshly prepared samples (some error bars are too small to visualize).

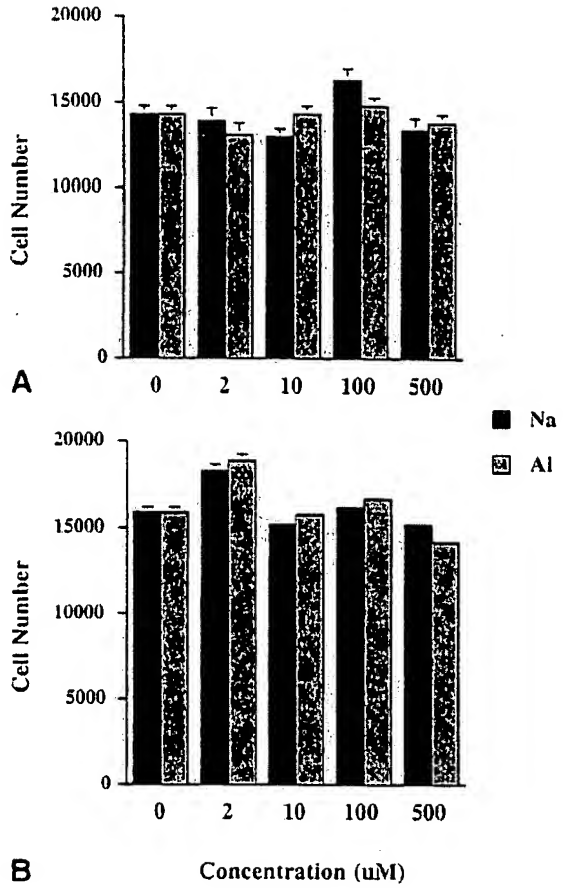

Fig. 2. Cell proliferation in human T98G cells treated with different concentrations of aluminum and sodium sulfate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean ± S.E. ($n = 12$). Two to three experiments were conducted on separate days with freshly prepared samples (some error bars are too small to visualize).

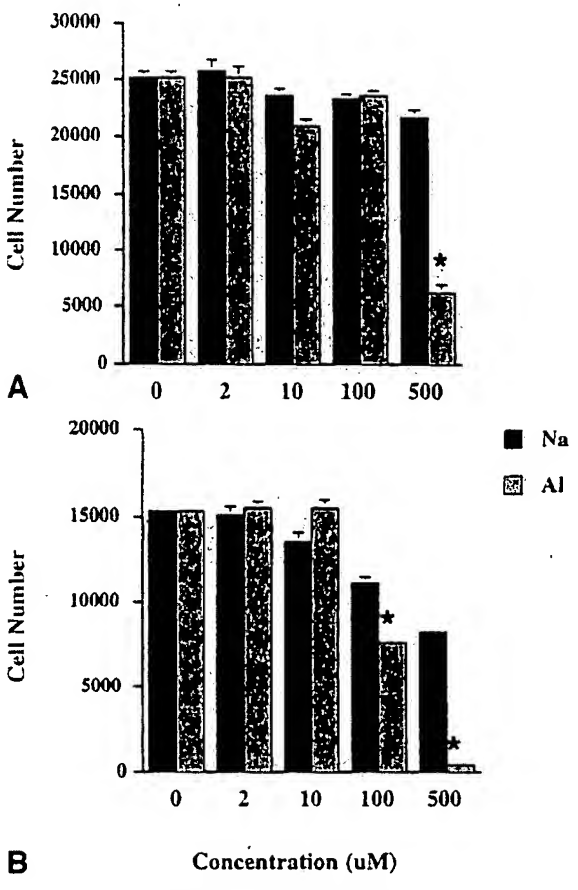

Fig. 3. Cell proliferation in human SK-N-SH cells treated with different concentrations of aluminum and sodium acetylacetonate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean ± S.E. ($n = 12$). Two to three experiments were conducted on separate days with freshly prepared samples. (*) Value is significantly different ($P < 0.001$) than the corresponding value using the sodium salt (some error bars are too small to visualize).

surface. Once detached, the ability of the cells to grow is compromised. SK-N-SH cells exposed to the same concentration of sodium acetylacetonate for 48 h showed a lesser decline in the number of attached cells (Fig. 3). The T98G cells were more resistant to the effect of aluminum acetylacetonate and sodium acetylacetonate on cell detachment, in that a 48 h exposure to 100 μM of the salts did not significantly alter the number of the attached cells (Fig. 4).

Effect of Aluminum on Cell Viability

After 48 h of treatment with 500 μM of aluminum acetylacetonate, few cells remained attached. Among the detached cells, 55% of the SK-N-SH cells were viable while 28% of the T98G cells were alive (Fig. 5). Thus, although cell proliferation was reduced to a greater extent in the SK-N-SH cells and these cells were more prone to becoming separated from the growth surface following exposure to aluminum acetylacetonate, once detached, the T98G cells were more sensitive to the toxic effect of the aluminum salt. Aluminum sulfate had no significant toxicity at the same concentration and time points assayed. Therefore, the acetylacetonate anion was necessary for the ability of aluminum to elicit an effect on the human cell lines.

Effect of the Addition of Phosphate on Cell Attachment

To determine whether phosphate would protect cells against the detachment and subsequent decrease in viability caused by aluminum acetylacetonate, 3 mM of sodium phosphate was added concomitantly with 500 μM of the Al salt. The addition of phosphate fully

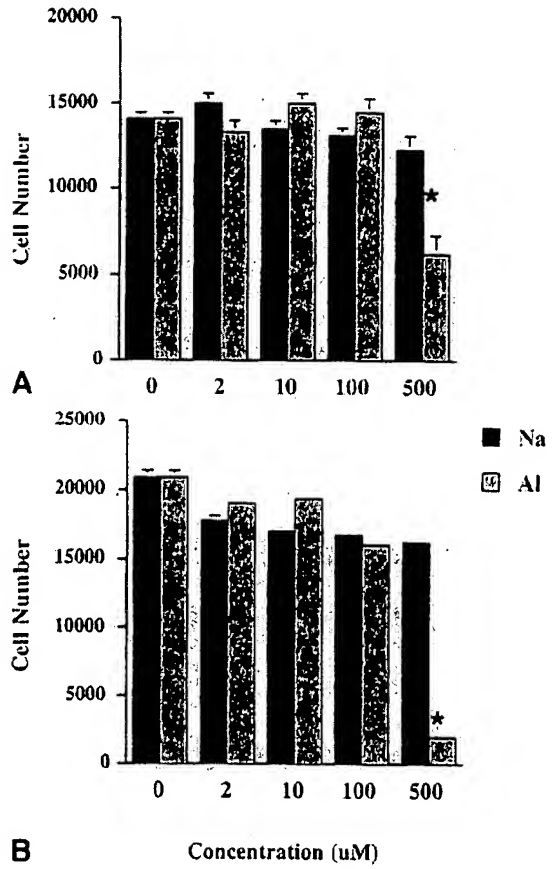

Fig. 4. Cell proliferation in human T98G cells treated with different concentrations of aluminum and sodium acetylacetonate. (A) Cells treated for 4 h. (B) Cells treated for 48 h. Values are mean ± S.E. ($n = 12$). Two to three experiments were conducted on separate days with freshly prepared samples. (*) Value is significantly different ($P < 0.001$) than the corresponding value using the sodium salt (some error bars are too small to visualize).

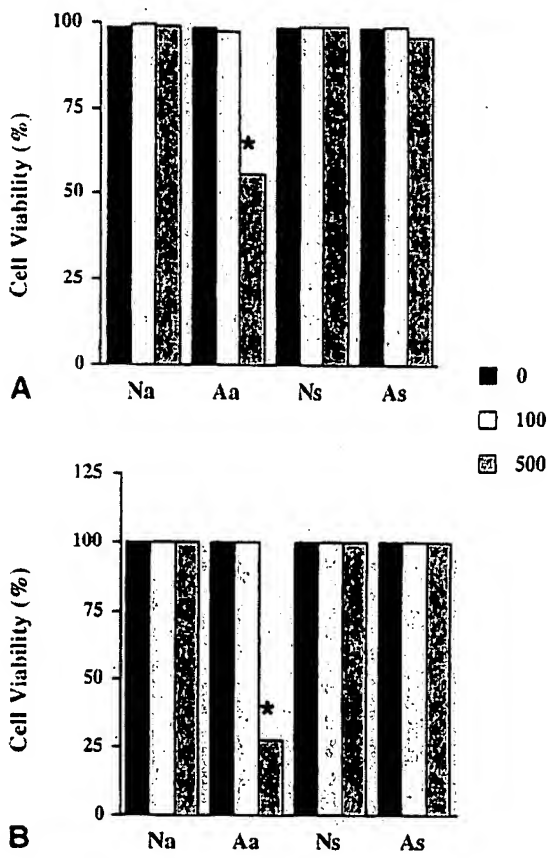

Fig. 5. Viability of human neuroblastoma SK-N-SH (A) and human glioblastoma T98G (B) cells treated with different concentrations of aluminum and sodium salts for 48 h (Aa: aluminum acetylacetonate; Na: sodium acetylacetonate; As: aluminum sulfate; Ns: sodium sulfate) (error bars are too small to visualize).

protected the T98G cells against aluminum acetylacetonate-induced cell detachment and subsequent death, but did not protect, even partially, the SK-N-SH cells (Fig. 6). Since only the attached cells were studied, and all of the SK-N-SH cells were detached from the plate, the viability is indicated as zero. This meant that there were no cells remaining in the well.

Intracellular Aluminum Content

Aluminum acetylacetonate, at high concentrations, was toxic to cell lines of neural origin. Since aluminum sulfate was not toxic to the cells at any of the concentrations tested, it was postulated that the lipophilic nature of aluminum acetylacetonate increased the rate of absorption of the metal into cells. To determine cellular uptake of Al, 20 μM of the morin dye was incubated with washed cell lysates and the fluorescence was monitored. The addition of the morin dye to the treated samples did not produce significant fluorescence (Table 3).

Table 3
Al-morin complex formation in Al-treated cells[a]

	Al-morin complex formation after 1 h (arbitrary units of fluorescence) ± S.E.
(A) T98G cells	
Control	1.27 ± 0.52
$Al(acac)_3$	1.88 ± 0.32
$Na(acac)_3$	0.02 ± 0.30
(B) SK-N-SH cells	
Control	0.65 ± 0.54
$Al(acac)_3$	0.68 ± 0.59
$Na(acac)_3$	0.63 ± 0.40

[a] Al-morin binding in Al-treated cell lines. Cells were treated for 48 h with 100 μM of aluminum or sodium acetylacetonate. (A) Human glioblastoma (T98G) cells. (B) Human neuroblastoma (SK-N-SH) cells. Values are mean ± S.E. Each group represents results from three separate experiments ($n = 4$–6).

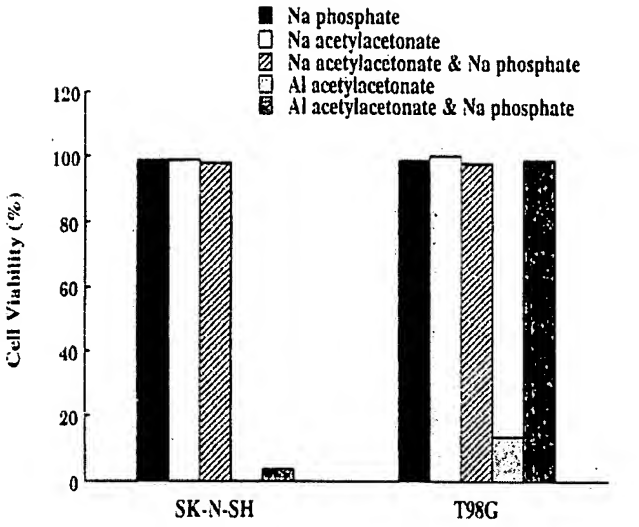

Fig. 6. The effect of the addition of 3 mM sodium phosphate simultaneously with 500 μM of either sodium or aluminum acetylacetonate on the viability of human neuroblastoma SK-N-SH and human glioblastoma T98G cells. Cells were treated for 48 h (error bars are too small to visualize). In this figure, only the attached cells are analyzed. The viability of SK-N-SH cells is indicated as zero because all of the cells were detached and none remained in the well.

Since morin-Al complex formation depends on the presence of positively charged aluminum species, it was postulated that the aluminum may be bound to cellular components and thus cannot associate with the morin dye. Therefore, samples of the treated cells were sent to a commercial laboratory, Desert Analytics (Tucson, AZ), for the determination of Al content by atomic absorption spectroscopy. However, there was high variability in the results and there was no significant change apparent in Al content in the control and treated cells (data not shown).

DISCUSSION

Human cell lines constitute a useful model for determining the mechanisms responsible for the toxicity of exogenous compounds. This system provides a homogenous population of cells where the effect of a compound on a specific cell type can be assessed. In the present study, two biologically relevant aluminum salts were studied to determine neurotoxicity. Aluminum sulfate is found in drinking water and antiperspirants while aluminum acetylacetonate is an analog of a neutral Al complex formed with isomaltol, a starch byproduct, in the GI tract (Powell and Heath, 1996). Treatment of cells with inorganic aluminum sulfate did not change proliferation or viability in either of the two human cell lines. This finding was paralleled by earlier results using rodent cell cultures (Campbell et al., 1999). However, aluminum acetylacetonate showed time and dose-dependent toxicity. At high concentrations, both aluminum sulfate and aluminum acetylacetonate form positively charged Al complexes. It is possible that these charged Al species interact with negatively charged proteins on the surface of the cell membrane or bind to phosphate groups in the phospholipid bilayer and by doing so compromise the integrity and function of the cell membrane. However, since aluminum sulfate did not cause adverse effects in the cells, the lipophilic nature of the salt may be necessary for this interaction.

The binding of Al to membrane phospholipids was reported in a study, which showed that membranes containing phosphatidyl serine and dipalmitoylphosphatidylcholine showed significant rigidification after treatment with aluminum (Deleers et al., 1986). It has also been demonstrated that aluminum can cause the formation of negatively charged phospholipid clusters which then decrease the mobility of fatty acids (Verstraeten et al., 1997) and this may underlie the rigidity seen in Al-treated membranes. The exposure of human erythrocytic membranes to aluminum acetylacetonate changed the conformation of membrane proteins (Zatta et al., 1997) and modified the discoid shape of erythrocytes by interacting with both the inner and outer membrane of the cells (Suwalsky et al., 1999). It is possible that the difference in the sensitivity of the two different cell lines to the organic aluminum salt is due to the dissimilarity between their membrane composition. The capability of Al to bind strongly to phosphate groups may underlie its potential to bind to the cellular phospholipid bilayer and disrupt the integrity of the membrane. The addition of 3 mM sodium phosphate was able to completely reverse the effect of aluminum acetylacetonate on the glioblastoma cells. However, it did not alter the response of neuroblastoma cells to the aluminum salt. This difference suggests distinct mechanisms of Al toxicity in the two cell lines.

Neurofibrillary tangles (NFT) are composed of paired helical filaments (PHF) formed by abnormally phosphorylated human tau (PHFτ or A68) protein (Shin et al., 1995). The presence of aluminum has been demonstrated in NFT present in cases of AD and elderly controls while healthy neurons were reported not to contain the metal (Perl and Brody, 1980). Circular dichroism and NMR spectroscopy studies have shown that aluminum does indeed bind to tau and by doing so induces the aggregation of the protein (Madhav et al., 1996). Co-injection of aluminum with

PHFτ results in aggregates that last longer than the deposits that are usually formed by the injection of PHFτ alone (Shin et al., 1994). Al-induced aggregation of tau protein increases 3.5 fold upon phosphorylation of the protein and the aggregates formed in the presence of the metal are resistant to proteases and phosphatases (Li et al., 1998). Since aluminum may strongly bind to neuronal cell components, this may explain why the addition of phosphate does not have a protective effect on aluminum acetylacetonate induced cell detachment. Since the SK-N-SH cells were not differentiated before addition of aluminum and the metal is known to interact with neurofilament proteins and tau, it would be interesting to study the effect of the metal on differentiated, post-mitotic neuronal cells.

Interactions of Al with membrane components seem to be an important factor in Al-induced toxicity, and glial cells appear to be more susceptible to the toxic effect of the salt. The increased susceptibility of glial cells rather than neurons is in accord with a report that primary glial cells are more vulnerable than neurons to long term exposure to aluminum (Suárez-Fernández et al., 1999). Al induces apoptosis only in cultured astrocytes and not neurons, and this provides yet another clue that neurodegeneration may indeed initially be due to the compromised state of the astroglial cells leading to the loss of viability and function of neuronal cells (Suárez-Fernández et al., 1999). It is possible that a stressor, such as increased levels of extracellular aluminum, may trigger the activation of glial cells. During aging, the cerebral microvasculature becomes more prone to damage and this may result in the compromise of the blood brain barrier (BBB) (Kemper, 1984). Since this barrier is the major mechanism by which the brain keeps out foreign antigens, jeopardizing the BBB could lead to compounds, such as aluminum, which are generally confined to the systemic circulation to enter the brain. Cerebral levels of aluminum have in fact been found to increase with age (McDermott et al., 1979). Once Al is accumulated in the brain, it can activate microglia which then play an important role in neurodegeneration by initiating inflammation and secreting complement proteins and oxygen radicals which can then kill healthy neurons and ultimately lead to dementia (Campbell and Bondy, 2000).

ACKNOWLEDGEMENTS

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of: ROSENTHAL et al.	Group Art Unit: 1614
Serial No.: 10/728,277	Examiner: Roberts, Lezah
Filed: December 4, 2003	RULE 132 DECLARATION OF ANTONY JAMES MATHEWS (37 C.F.R. § 1.132)
Conf. No.: 7142	
Atty. File No.: 42830-10010	
For: "TREATMENT OF MUCOSITIS"	

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir or Madam:

I, Antony James Mathews, residing at 2938 Kalmia Avenue #5, Boulder, CO 80301, USA, declare as follows:

I. Qualifications And Basis For Declaration:

I am currently employed by Endo Pharmaceuticals Colorado, Inc., formerly named RxKinetix, Inc., as Director, Formulation Development. I have significant experience working with poloxamer 407 and formulating compositions including poloxamer 407. The attached **Appendix A** is a detailed summary of my technical qualifications.

I have reviewed and considered an Office Action dated January 5, 2007 issued by the United States Patent and Trademark Office (the "**Office Action**") concerning the Pending Application, a copy of which is included in **Appendix B**.

I have reviewed and considered U.S. Patent Number 6,503,955 by Dobrozsi et al. ("**Dobrozsi et al.**"), which was cited in the Office Action. A copy Dobrozsi et al. is included in **Appendix C**:

I have reviewed and considered the claims that are set forth in **Appendix D**.

I also have performed some tests on viscosity behavior on example compositions within the disclosure of Dobrozsi et al., and summaries of those tests are provided in **Appendix E.**

II. Excerpts Of Teachings Of Dobrozsi et al.

The following are some excerpts from Dobrozsi et al. These excerpts are each assigned reference numbers beginning with "D." In the discussion that follows in section III of this Declaration, I make reference as appropriate to various ones of these excerpts to identify portions of Dobrozsi et al. relevant to the discussion. Such References to these excerpts are made in brackets in the body of the discussion below using the assigned reference numbers.

D1. Column 2, lines 18-67.

> Attempts to develop such compositions have been ongoing for a significant period of time. Examples of such compositions include intra-ocular dosage forms as disclosed in Edsman, K., Carlfors, J., Petersson, R., *Rheological Evaluation of Poloxamer as an In Situ Gel for Ophthalmic Use*, European Journal of Pharmaceutics Vol. 6 pp.105-112 (1998) herein incorporated by reference. Compositions such as these are broadly described as primarily aqueous solutions of block co-polymer surfactants, other wise referred to as "poloxamers", that are commonly known in the art. When formulated in water as somewhat concentrated solutions, or with water and co-solvents, the poloxamer solution remains as a pourable liquid. The most commonly reported example of this type of system consists of poloxamer 407 at concentrations ranging from about 10% to 35% by weight of the composition in water. These compositions are administered at room temperature as liquids. They form a gel upon reaching body temperature. The trigger for converting these compositions to a gel, therefore, is body heat.
>
> In situ gelation of pharmaceutical compositions based on poloxamer that are biologically triggered are known in the art. For example Kim, C. K., Lee, S. W., Choi, H. G., Lee, M. K., Gao, Z. G., Kim, I. S., and Park, K. M.: *Trials of In Situ Gelling and Mucoadhesive Acetaminophen Liquid Suppository in Human*

Subjects, International Journal of Pharmaceutics vol. 174, pp. 201-207 (1998) incorporated herein by reference. Kim et al. discloses liquid suppositories for enhancing absorption of the pain and fever relieving drug acetaminophen.

U.S. Pat. No. 5,256,396, issued Oct. 26, 1993, to Colgate Palmolive Company, incorporated herein by reference, describes similar compositions containing poloxamer 407 and water at specified concentrations. Other products utilizing bio-triggers include those comprising poloxamer 407 at ranges preferably 12% to 17%. When combined with pharmaceutically active agents, these compositions are injected into the gingival space between the root of a tooth and the gum.

Poloxamers represent a large family of polymers that vary in molecular weight as well as in the percentage or portion of the block copolymer that is considered hydrophobic. Compositions comprising other poloxamers from this family having similar liquid/gelling characteristics are somewhat predictable, lacking only in the understanding of the required concentration of poloxamer. While there is a large number of uses for such compositions, they all rely on the same general mechanism of temperature-induced gelation of aqueous poloxamer dispersions. Compositions known in the art are found to be inadequate, however, as the gel structure readily dissolves in aqueous environments.

D2. Column 3, lines 2-21.

The present invention covers pourable liquid vehicles used to deliver compositions, materials and substances to moistened surfaces and aqueous environments. The benefits of compositions formulated with such pourable liquid vehicles include retention of the compositions, materials and substances on the moistened surface. This in turn allow for effective delivery of a desired composition, material and substance in the vehicle that acts on targeted surface, resisting erosion or run-off even in an aqueous environment. Such pourable liquid vehicles have a number of utilities for delivery of all kinds of materials including but not limited to cleaning and treating surfaces of objects as well as biological or

living organisms, including living creatures.

Another object of this invention is to utilize such pourable liquid vehicles to deliver health care compositions and materials and substances to living creatures, particularly mammals, and most particularly humans. Even another object of the present invention is to develop a method for effective delivery of health care compositions, materials and substances.

D3. Column 3, lines 32-47.

The term "pourable liquid" as used herein means the physical state of the compositions of the present invention prior to formation of a gel.

The term "moistened surface" as used herein means any living or non-living surface having sufficient moisture in or on it to trigger rapid conversion of a pourable liquid to a gel.

The term "in situ gelation" as used herein means the conversion of a pourable liquid to a gel at a designated site or surface.

As used herein, the term "gel" describes the substance resulting from the combination of the pourable liquid and water, or bodily fluid containing mostly water. The gel is sufficiently viscous to remain at the site applied to, or ultimately targeted for, over a period of time sufficient for the compositions, materials and substances in the gel to bring about a desired result at the site they are delivered to.

D4. Column 3, line 65 through column 4, line 18.

The "viscosity" of a viscous material, also called viscosity index, is defined as the ratio of the shear stress applied into the material, divided by the rate of shear which results. Materials of a higher viscosity have a higher resistance to flow, or to forces which can induce flow, than a lower viscosity material. All viscosities listed herein are at a shear rate of about 50 per second unless otherwise indicated. All of the rheologic characteristics given herein can be measured in a

controlled rate or a controlled stress rotational viscometer capable of some operation in a controlled rate mode, for Example Haake RS 150 by Haake GmbH, Karlsruhe, Germany; Carrimed CSL 500 Controlled Stress Rheometer by TA Instruments, New Castle, Delaware; and Rheometric SR5, by Rheometric Scientific, Piscataway, N.J.

Specifically, when subject to constant shearing rate of about 50 per second at normal ambient temperature (approx. 25° C.), the present liquid compositions have a viscosity of less than about 7 pascal seconds, preferably less than about 2 pascal seconds, more preferably less than about 1 pascal seconds.

D5. Column 4, lines 19-32.

The value of a composition's triggered viscosity ratio ("T") is useful in determining the degree to which a composition exhibits the above described gelling characteristic. The formula and procedure for determining the triggered viscosity ratio is set forth below.

It is desirable for the compositions of the present invention to exhibit a triggered viscosity ratio of at least about 1.3, preferably at least about 2, more preferably at least about 5, and most preferably at least about 10 wherein the triggered viscosity is defined by the following formula or ratio:

$$T = \eta_g / \eta_f$$

where η_g=viscosity of the gel and

where η_f=viscosity of the pourable liquid

D6. Column 4, lines 33-48.

The pourable liquid vehicle of the present invention must be selected and formulated so that the contacting and mixing said vehicles to a mucosal surface of the body, or with some other fluid in the body, triggers the conversion of the pourable liquid vehicle to a more viscous gel-like mixture. Examples of these fluids are saliva, gastric fluid, intestinal fluid, extracellular fluid present under the

skin at the site of a subcutaneous injection, or in muscle tissue at the site of an intramuscular injection, cerebrospinal fluid, vaginal fluid, fluid exudate from an open wound or ulcer, tear fluid, rectal fluid, or any other bodily fluid of an animal which contains in large measure water. In other words, after the pourable liquid vehicle contacts with the bodily fluid, the viscosity of the pourable liquid vehicle becomes greater than the viscosity of either the pourable liquid vehicle itself prior to mixing, or the bodily fluid alone.

D7. Column 4, line 33 through column 5, line 33.

The triggered viscosity ratio of a pourable liquid vehicle can be determined by one skilled in the art using appropriate viscosity measuring instruments, and is exemplified by the following method. First, the viscosity of the pourable liquid vehicle (η_f) is determined in a rheometer using a shear rate of 50 per second at 25° C. For the determination of η_f, 1 ml of the pourable liquid vehicle is placed onto the plate of a Haake RS 150 rheometer. The temperature is controlled in the range of typical room temperature, about 25° C. A cover is used on the measuring system and a solvent-saturated atmosphere provided to prevent evaporation of water, ethanol, or other volatile components from the sample during the test. A 35 mm diameter parallel plate measuring system is lowered onto the sample, leaving a gap of about 1 millimeter, and an equilibration shearing of approximately 10 per second is applied for 10 seconds. Then, a constant shearing rate of 50 per second is applied for 30 seconds. The viscosity η_f is read from the instrument at the 30 second time point.

For the determination of η_g, two dilutions of the pourable liquid vehicle are made with water. The first dilution is made to contain 75% by weight of the pourable liquid vehicle, and 25% by weight of additional water. The second dilution is made to contain 50% by weight of pourable liquid vehicle and 50% by weight of additional water. The pourable liquid vehicle and water are combined in a vial and a tight seal applied to prevent evaporation of components. The vial contents are mixed in an unusual manner, by repeated centrifugation. This is

necessary since some of the combinations are very viscous gels. Specifically, the vials are centrifuged (using for example a Beckman GS-6R centrifuge, available from Beckman Instruments, Palo Alto, Calif.) 20 minutes at 3000 RPM and 25° C. for at least four separate centrifuge runs. After each run the vials are inverted. Additional runs are conducted in the centrifuge to ensure complete mixing. 1 ml of the gelled sample is then loaded onto the plate of the same rheometer used for the measurement of η_f, except that the temperature is controlled at the normal body temperature of a human, 37° C. An identical rheometer measurement program is used as for determination of η_f. The triggered viscosity factor for both the 25% and 50% dilution of the sample is calculated from η_f and η_g as described by the formula above. These two dilutions have been found to be useful for measuring the gelling functionality of the pourable liquid vehicles of the invention in a standardize method, because some of the pourable liquid vehicles may require a greater or lesser amount of water in order to trigger the gelling character. The use of other water dilutions for determination of η_g, ranging from about 5% up to about 70%, would also be expected to provide a demonstration of the unique, gelling character of the invention, but the dilution which yields a maximal value of T varies depending upon the exact pourable liquid vehicle being tested.

D8. Column 5, lines 34-47.

All percentages of the components comprising the invention are herein referred to their weight in the pourable liquid vehicle as a whole.

The present invention is a pourable liquid vehicle comprising:

(a) from about 26% to about 100% polyoxyalkylene block copolymer;

(b) from about 0% to about 70% glycol; and

(c) from about 0% to about 50% water;

wherein said vehicle is used to deliver compositions, materials and substances to moistened surfaces and aqueous environments said vehicle has a viscosity value

η_f less than or equal to 7 pascal-seconds and the value T greater than or equal to about 1.3.

D9. Column 5, lines 51-54.

Polyoxyalkylene block copolymers herein referred to as "poloxamers" are nonionic block copolymers of ethylene oxide and propylene oxide corresponding to the following structure:

D10. Column 6, lines 61-63.

Preferred glycols are selected from the group consisting of ethanol, glycerol and propylene glycol, and mixtures thereof.

D11. Column 6, line 66 through column 7, line 4.

In addition to the poloxamers, and, or the glycol, it is desirable in some of the pourable liquid vehicles of the present invention to include water. Water is useful at a level from 0% to about 50%, preferably about 1% to about 46%, most preferably from about 2% to about 41% of the pourable liquid vehicle.

D12. Column 7, lines 7-25.

Preferred embodiments of the present invention utilizing the combination of poloxamers, polyols and water include the following:

1. from about 26% to about 65% Pluronic F127, from about 22% to about 38% ethanol and from about 8% to about 45% water.
2. from about 52% to about 60% Pluronic F108, from about 20% to about 25% ethanol and from about 17% to about 27% water.
3. from about 25% to about 50% Pluronic P105, from about 45% to about 65% propylene glycol and from about 5% to about 20% water.

4. from about 37% to about 77% Pluronic P105, from about 12% to about 28% ethanol, and from about 10% to about 45% water

5. from about 26% to about 49% Pluronic F127, from about 2% to about 12% ethanol, from about 30% to about 68% propylene glycol, and from about about [sic] 7% to about 40% water.

III. The Teachings Of Dobrozsi et al. In Relation To Claims In Appendix D .

Claim 1, as set out in Appendix D, identifies a composition useful for treatment of oral mucositis. According to Claim 1, the composition must include at least the three identified components in a particular formulation, namely: N-acetylcysteine, poloxamer 407 in an amount of 5 to 20 weight percent of the composition, and carrier liquid comprising water in an amount sufficient as formulated in the composition to interact with the poloxamer 407 to impart reverse-thermal viscosity behavior. Also, the composition exhibits reverse-thermal viscosity behavior over at least some range of temperatures between 1°C and 37°C, and when at some reduced temperature in the range of 2°C to 8°C the composition is an aqueous solution with both the poloxamer 407 and the N-acetylcysteine dissolved in the water.

Reverse-thermal viscosity is a property that is not common in liquid materials. The common effect of changes in temperature on the viscosity of liquids (including most liquid solutions) is that the viscosity of the liquid decreases with increasing temperature. Some aqueous solutions of poloxamer 407, however, are anomalous because at some temperatures the solutions exhibit a viscosity increase with increasing temperature. This uncommon type of viscosity behavior is known as "reverse-thermal", because the relationship between temperature and viscosity is a reverse of that which is most common for liquid formulations. Some, but not all, aqueous solutions of poloxamer 407 exhibit reverse-thermal viscosity behavior at some temperatures between 1°C and 37°. Some factors affecting whether a particular aqueous solution of poloxamer 407 would exhibit reverse-thermal viscosity behavior include the concentration of poloxamer 407 and the nature and concentrations of other components in the solution. Determining whether any particular aqueous solution of poloxamer 407 exhibits reverse-thermal viscosity behavior within any particular temperature range is a simple matter of making the

solution and testing the viscosity of the solution at various temperatures within that range, which is well within the capabilities of an ordinarily skilled chemist.

Dobrozsi et al. disclose what they call a "pourable liquid vehicle", which they disclose as being useful for delivering materials to moistened surfaces and aqueous environments, where the delivered materials are retained at the moistened surface [D2]. Dobrozsi et al. provide an extensive listing (at column 7, line 26 through column 9, line 27) of possible materials that might be delivered using the pourable liquid vehicle.

Dobrozsi et al. disclose that a property of their pourable liquid vehicle is that it forms a gel when it combines with water or bodily fluid containing mostly water, and that the gel that forms is sufficiently viscous to remain at the site of application, to retain the delivered material at that site [D2, D3, D6]. It is the contacting and mixing of the pourable liquid with water or such bodily fluid that causes, or triggers, the conversion of the pourable liquid vehicle to the gel form [D6].

The pourable liquid vehicle of Dobrozsi et al. has a viscosity of less than 7 pascal seconds, and more preferably smaller than 1 pascal second [D4, D8]. A viscosity of 7 pascal seconds is equal to 7000 centipoises (cP). The viscosity of the gel formed from the pourable liquid vehicle has a viscosity at least 1.3 times, and most preferably 10 times, as large as the viscosity of the pourable liquid vehicle [D5]. Dobrozsi et al. refer to the ratio of the viscosity of gel to the viscosity of the pourable liquid vehicle as the "triggered viscosity ratio" [D5]. The triggered viscosity ratio is dependent upon the degree of dilution to which the pourable liquid is subjected, and Dobrozsi et al. describe determination of this triggered viscosity ratio for any given degree of dilution by measuring the viscosity of that pourable liquid vehicle at 25°C (typical room temperature), diluting the pourable liquid vehicle with water by the desired amount to form a gel (Dobrozsi et al. suggest doing dilutions at 25:75 and 50:50 parts water to parts pourable liquid vehicle), measuring the viscosity of the resulting gel at 37°C (human body temperature), and dividing the measured viscosity of the gel by the measured viscosity of the pourable liquid vehicle [D7].

On page 4 of the Office Action, the patent Examiner asserts that the viscosity of the pourable liquid vehicle of Dobrozsi et al. was shown to increase with an increase in temperature from room temperature to 37°C. The Examiner's exact statement is quoted as follows:

> The pourable liquid vehicle of the disclosed invention [of Dobrozsi et al.] were formulated so that the contacting and mixing said vehicles to a mucosal surface of the body, or with some other fluid in the body, triggers the conversion of the pourable liquid vehicle to a more viscous gel-like mixture (col. 4, lines 33-48). The viscosities of the formulated vehicles were measured at room temperature and 37°C the temperature inside the human body. It was disclosed the viscosity of the compositions increased at the higher temperature, therefore encompassing claim 1. [Emphasis as in original.]

The Examiner's reference to the higher viscosity at 37°C relative to the viscosity at room temperature must relate to that portion of Dobrozsi et al. describing the determination of the triggered viscosity ratio, which, as discussed above, involves measurement of the viscosity at 25°C of the pourable liquid and measurement of the viscosity at 37°C of the gel that forms after dilution of the pourable liquid vehicle. This portion of Dobrozsi et al. is quoted in excerpt D7 above. However, that portion of Dobrozsi et al. does not describe a reverse-thermal viscosity behavior for the pourable liquid vehicle of Dobrozsi et al. As discussed above, Dobrozsi et al. describe the conversion of the pourable liquid vehicle to a gel as being caused by dilution of the pourable liquid with water or certain bodily fluids, not by temperature change. It seems apparent that the reason Dobrozsi et al. measure the temperature of the gel at 37°C is simply because that is the temperature to which a composition would be subjected in a human body following administration. Regardless that Dobrozsi et al. measure the viscosity of the pourable liquid vehicle and the gel at different temperatures, the disclosure of Dobrozsi et al. is that formation of the gel is caused by dilution. Formation of a gel caused by dilution of the pourable liquid vehicle as disclosed by Dobrozsi et al. is not reverse-thermal viscosity behavior.

Moreover, reverse-thermal viscosity behavior is a property of a material exhibited when the material is subjected to change in temperature, all other variables remaining constant. As a property of a material, reverse-thermal viscosity behavior does not involve a compositional change to the material. The conversion of the pourable liquid vehicle to a gel as disclosed by Dobrozsi et al., however, is based not on a temperature effect on viscosity, but upon a compositional change that occurs when the pourable liquid vehicle is diluted with water or certain bodily fluids. The gel described by Dobrozsi et al. is a different composition than the pourable liquid vehicle, due to the addition of the dilution liquid. Comparing the viscosity of the

gel at one temperature to the viscosity of the pourable liquid vehicle at a different temperature provides no information about the effect of temperature on the viscosity of either the gel or the pourable liquid, because they are different compositions. Claim 1, as set forth in Appendix D, describes a composition having a property of reverse-thermal viscosity behavior (i.e., that the viscosity of the composition increases with increasing temperature of the composition). Reverse-thermal viscosity behavior is significantly different than what Dobrozsi et al. describe, which is a change of composition through dilution of the pourable liquid vehicle to form a new composition (containing the dilution liquid) that is in the form of a gel. The conversion of the pourable liquid to a gel with a change in composition is not reverse-thermal viscosity behavior.

Dobrozsi et al. make reference to reverse-thermal viscosity behavior only in the background section of that document, with the reference being to certain compositions made using poloxamers [D1]. Concerning those background compositions, Dobrozsi et al. recognize that they have a reverse-thermal gelation property, stating:

> These compositions are administered at room temperature. They form a gel upon reaching body temperature. The trigger for converting these compositions to a gel, therefore, is body heat. [D1, emphasis added.]

Dobrozsi et al. then assert that these previously known poloxamer compositions are inadequate, and state:

> While there is a large number of uses for such compositions, they all rely on the same general mechanism of temperature-induced gelation of aqueous poloxamer dispersions. Compositions known in the art are found to be inadequate, however, as the gel structure readily dissolves in aqueous environments. [D1, emphasis added.]

As discussed above, Dobrozsi et al. disclose that formation of a gel from their pourable liquid vehicle is triggered not by heat, but by a compositional change, i.e., dilution with water or certain bodily fluids. It is clear that Dobrozsi et al. are not relying on reverse-thermal viscosity behavior as a mechanism for gel formation, and Dobrozsi et al. are proposing their pourable liquid vehicle as a superior alternative to those previously known compositions that rely on

reverse-thermal viscosity behavior for gel formation. Gel formation through viscosity increase caused through compositional change by the addition of a triggering component, e.g., aqueous dilution fluid added to the pourable liquid vehicle in Dobrozsi et al., involves a fundamentally different mechanism for viscosity increase than the mechanism of reverse-thermal viscosity increase, which is caused simply by an increase in temperature. Making a drug delivery formulation including reverse-thermal viscosity behavior would not be obvious from the teaching of Dobrozsi et al., which finds such formulations as inadequate and offers an alternative to address such inadequacy.

It is true that Dobrozsi et al. disclose that one of the components of their pourable liquid vehicle is a poloxamer, which Dobrozsi et al. refer to as "polyoxyalkylene block copolymers" [D9]. But it is clear that Dobrozsi et al. are not using the poloxamer for its ability to be formulated to make compositions exhibiting reverse-thermal viscosity behavior and, as discussed above, not all poloxamer formulations exhibit such reverse-thermal viscosity behavior.

Dobrozsi et al. require that their pourable liquid vehicle contains 26 weight percent to 100 weight percent polyoxyalkylene block copolymer, and optionally up to 70 weight percent glycol and up to 50 weight percent water [D8]. Moreover, Dobrozsi et al. require that within these compositional constraints, the pourable liquid vehicle must also be formulated so that contacting and mixing the pourable liquid vehicle with a body fluid containing mostly water causes the formation of a gel [D3, D6]. Dobrozsi et al. list as preferred embodiments for the pourable liquid vehicle certain compositions including "Pluronic F127" [D12], which is a poloxamer 407, the type of polymer specified in Claim 1 as set forth in Appendix D. These preferred compositions include at least 26 weight percent Pluronic F127 and either ethanol and water in certain concentrations or ethanol, propylene glycol and water in certain other concentrations [D12].

Based on a consideration of the teachings of Dobrozsi et al. and my experience with formulations including poloxamer 407, e.g., Pluronic F127, I expected that many, if not most, of the pourable liquid vehicle compositions described by Dobrozsi et al. would not exhibit reverse-thermal viscosity behavior of the type as recited in Claim 1 of Appendix D. Many of the possible compositions for pourable liquid vehicle within the teachings of Dobrozsi et al. contain 100% poloxamer, while others contain only poloxamer and a glycol, and none of the possible compositions for the pourable liquid vehicle contain more than 50% water.

To test my expectation, I prepared some example compositions that would be within the teachings of Dobrozsi et al. for the pourable liquid vehicle to test their viscosity behavior. Table 1 below summarizes four different test compositions that I prepared for testing, all including poloxamer 407. A summary of the preparation and viscosity testing of each of the these example compositions is included in Appendix E.

TABLE 1

Example No.	Poloxamer 407 weight %	Water weight %	Ethanol weight %	Propylene Glycol Weight %	Viscosity Behavior
1	26	50	24	--	No reverse-thermal viscosity behavior
2	26	50	--	24	Reverse-thermal viscosity behavior, but extremely high viscosity unsuitable as pourable liquid vehicle of Dobrozsi et al., which must have viscosity of less than 7000 Cp
3	26	37.1	36.9	--	No reverse-thermal viscosity behavior
4	26	37.1	--	36.9	Could not successfully prepare a homogenous mixture for testing

For all example compositions, poloxamer 407 was used at a concentration of 26 weight percent, because that is the minimum concentration of poloxamer 407 permitted by Dobrozsi et al. [D8, D12] and is closest to the range of poloxamer 407 concentrations required in Claim 1 of Appendix D (5 to 20 weight percent). All tests included water in an amount so that in all example compositions water is the largest liquid component, so that all example compositions would be aqueous solutions, because an aqueous solution is a requirement of Claim 1 of Appendix D. Two example compositions (Examples 1 and 2) used 50 weight percent water, because that is the maximum water concentration permitted by Dobrozsi et al. [D8,D11]. Two other example compositions (Examples 3 and 4) used 37.1% water, as a minimum amount of water that could be used in combination with another liquid component to make a solution that would be aqueous. The remaining portion of each example composition was then made up of

either ethanol (Examples 1 and 3) or propylene glycol (Examples 2 and 4). Ethanol was used because it is specified by Dobrozsi et al. for use in preferred compositions made using poloxamer 407 [D12] and it is used extensively with poloxamer 407 (Pluronic F127) in the specific technical examples disclosed by Dobrozsi et al. in columns 9-17. Propylene glycol was used because it is listed by Dobrozsi et al. as a preferred glycol for use to make the pourable liquid vehicle [D10], and because it was used extensively in the technical examples presented by Dobrozsi et al. in columns 9-17.

Except for Example 4, after preparation, each of the example compositions were tested for viscosity behavior over a temperature range of 0°C to 40°C, which covers the 1°C to 37°C range of Claim 1 in Appendix D. I was unable after significant effort to obtain a homogeneous mixture of Example 4 suitable for viscosity testing, and that example composition was not tested for viscosity behavior. A brief summary of the results of the viscosity testing is presented in Table 1. A more extensive discussion of the test procedures and results for each of the examples is presented in Appendix E.

In summary, neither of the example compositions containing ethanol (Examples 1 and 3) exhibited reverse-thermal viscosity over any range of temperatures between 0°C and 40°C. (See, Figures 1 and 3, respectively, in Appendix E.) The compositions of those examples exhibited the normal viscosity behavior of steadily decreasing viscosity with increasing temperature. The composition of Example 2, containing 50% water and 24% propylene glycol did exhibit reverse-thermal viscosity behavior. (See, Figure 2 in Appendix E.) It is noted, however, that the viscosity of the composition of Example 3 at all temperatures tested was over a million cP, or over two orders of magnitude higher than the maximum viscosity of 7000 cP specified by Dobrozsi et al. for their pourable liquid vehicle, and likewise the composition of Example 2 is not a flowable medium at a refrigerated temperature in a range of from 1°C to 10°C as required in Claim 142 of Appendix D and is not suitable for use as a mouthwash as required in Claim 149 of Appendix D.

The testing confirmed my expectation that at least many of the pourable liquid vehicle compositions proposed by Dobrozsi et al. would not exhibit reverse-thermal viscosity behavior. This is not surprising, because, as discussed above, the pourable liquid vehicles of Dobrozsi et al. were prepared to address problems identified by Dobrozsi et al. with prior compositions that exhibited reverse-thermal viscosity behavior, and Dobrozsi et al. teach away from using reverse-

thermal viscosity behavior as a mechanism for drug delivery, finding that mechanism to be inadequate, as discussed above.

All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. I understand that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of this patent application or any patent issuing thereon.

Respectfully submitted,

Date: 7-3-2007

By: _____
Antony James Mathews

APPENDIX A

TO

DECLARATION

OF

Antony James Mathews

Detailed Summary Of Technical Qualifications

Curriculum Vitae: Dr. Antony James Mathews

Date : May 30, 2007
Nationality : New Zealand
Home Address : 2938 Kalmia Avenue #5, Boulder, CO 80301, USA
Degrees Held : BSc, PhD

Biotechnology Experience

- Director of Formulation Development. Member of CMC team. Leading several direct reports in support of all formulation characterization and stability issues for drug manufacturing and scale up.

- Key member of manufacturing and process development troubleshooting teams. Project leader for a 15 member multidisciplinary team including engineers, scientists and management, reporting directly to senior management and which solved critical protein purification problems saving $200,000 US per month.

- Project leader for the development of platelet and reagent red cell preservation media. Achieved comprehensive resolution of the platelet project in nine months. Appointed to Deputy Director of Health Sciences in recognition of experience and mentoring of colleagues.

- Expert in protein purification from milligram to multi-gram scales for the support of research, pre-clinical studies, purification process development and troubleshooting. Extensive expertise in protein chemistry including protein folding, chemical modification and crosslinking, and detailed chemical analysis of proteins.

- Experienced in the development of biochemical assays based on chromatography, UV-visible spectroscopy, mass spectrometry and chemical kinetics, and used to support research, product development and manufacturing.

- Expert in biophysical chemistry techniques used in structure-function screening, particularly in the reaction kinetics and equilibrium measurements of protein-small molecule interactions and protein-protein interactions.

- Trained and managed biology, protein purification, and biophysical chemistry research groups ranging in size from a few research associates to large groups including several PhD scientists.

Research and Development Experience

October 2006 – Present: Director, Formulation Development, Endo Pharmaceuticals Colorado, Boulder, Colorado, USA
RxKinetix was acquired by Endo Pharmaceuticals of Chadds Ford, Pennsylvania in mid-October. My position and duties remain unchanged.

January 2006 – October 2006: Director, Formulation Development, RxKinetix Inc., Boulder, Colorado, USA.
Responsible for formulation development and analysis of pharmaceuticals, including assay development, biophysical measurements, and stability measurements.

October 2005 – December 2005: Postdoctoral research in the laboratory of Professor Tom Brittain, School of Biological Sciences, University of Auckland, Auckland, New Zealand.
In collaboration with Associate Professor Angela Fago, a visiting researcher from the University of Aarhus in Denmark, performed biochemical and biophysical investigations of ligand binding and electron transfer reactions of recombinant neuroglobin and cytochrome *c*. This work was undertaken while waiting for my USA O-1 visa prior to taking my position at RxKinetix.

May 2003 – September 2005: Director of Research and Development, ICPbio Limited, Auckland, New Zealand.
Directed the activities of a small R&D group in support of company goals, including bioprocess and protein purification development of regulated protein products and assay development. Provided support for internal customers and external research interactions, including supervision of an MPhil. student, co-authoring a successful multiple institution grant application to investigate automated milk protein fractionation, and establishing contract research for embryo storage products.

July 2002 - February 2003: Head of Biochemistry, Genesis Research and Development Corporation Limited, Auckland, New Zealand.
Managed research groups and troubleshooting for the identification of proteins and peptides in plant phloem sap using two dimensional electrophoresis, two dimensional HPLC, and mass spectrometry.

July 2001 - June 2002: Project Leader - BioStore, Genesis Research and Development Corporation Limited, Auckland, New Zealand.
Assembled and managed research group characterizing blood platelet storage solutions and storage solutions with improved preservation of the antigen profiles of reagent red blood cells. Identified key research approaches, arranged and managed external research contracts.

March 2000 - June 2001: Senior Research Scientist, working for Professor John S. Olson, Department of Biochemistry and Cell Biology, Rice University, Houston, Texas. Simplified synthesis of mixed metal hybrid hemoglobins. Measurements of apo-hemoglobin stability. Hemoglobin-NO chemistry.

April 1999 - February 2000: Research Fellow, working for Associate Professor Tom Brittain, School of Biological Sciences, University of Auckland, Auckland, New Zealand.

Protein purification and analysis for X-ray crystallography and NMR experiments. Mechanism of heme insertion during hemoglobin biosynthesis. Isolation of peripheral blood reticulocytes by isopycnic density gradient ultracentrifugation. NMR studies of heme disorder in adult and recombinant human embryonic hemoglobins. Purification and analysis of recombinant hemoglobins from yeast by ion exchange and size exclusion chromatography, chromatofocusing, and reversed phase HPLC.

September 1990 - August 1998: Research Scientist II and III at Somatogen, Inc., Boulder, Colorado (known as Baxter Hemoglobin Therapeutics since June 1998).
Key member of protein purification process development and troubleshooting teams. Assay development using numerous biophysical/biochemical techniques. Developed laboratory facilities. Trained and managed research groups studying protein biophysical chemistry and protein purification from milligram to tens of grams scale. Biophysical and chemical analysis of self-associating proteins. Structure-function screening of recombinant hemoglobins for product development. Protein engineering of hemoglobin to decrease reactivity towards nitric oxide. Site directed chemical crosslinking of hemoglobin.

December 1986 - July 1990: Postdoctoral Research Fellow, working for Professor John S. Olson, Department of Biochemistry and Cell Biology, Rice University, Houston, Texas.
Heme pocket structure-function relationships in recombinant human hemoglobins. Laser flash photolysis and stopped-flow rapid kinetics of ligand binding to hemoglobin and myoglobin. Isolation of inclusion bodies, protein purification and refolding.

February - November 1986: Postdoctoral Research, working for Professor Barry T. Nall, Department of Biochemistry and Molecular Biology, Medical School, University of Texas Health Science Center at Houston (now at UTHSC San Antonio).
Thermodynamic stabilities and folding kinetics of yeast cytochrome *c*. Denaturant and thermally induced unfolding of proteins.

University Education
BSc (1981, Chemistry and Biochemistry) and PhD (1986, Biochemistry) degrees were awarded by the University of Auckland, New Zealand.
Doctoral Research Topic : Chemically Modified Cytochromes *c*.
Doctoral Advisor : Associate Professor Tom Brittain.
Scholarships : New Zealand Universities Postgraduate Scholarship.

Publications

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US Patent 5,844,090, December 1, 1998.

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S.J. Hoffman, D.D. Looker, M.S. Rosendahl, G.L. Stetler, M. Wagenbach, D.C. Anderson, A.J. Mathews and K. Nagai
US Patent 5,844,089, December 1, 1998.

Hemoglobin-like Protein Comprising Genetically Fused Globin-like Polypeptides
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Hemoglobins as Drug Delivery Agents
D.C. Anderson and A.J. Mathews
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US Patent 5,744,329, April 28, 1998.

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Hemoglobins as Drug Delivery Agents
D.C. Anderson and A.J. Mathews
US Patent 5,679,777, October 21, 1997.

Production and Use of Multimeric Hemoglobins
D.C. Anderson, A.J. Mathews and G.L. Stetler
US Patent 5,599,907, February 4, 1997.

APPENDIX B

TO

DECLARATION

OF

ANTONY JAMES MATHEWS

Copy of Office Action Dated January 5, 1007

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/728,277	12/04/2003	Gary J. Rosenthal	42830-10010	7142

25231 7590 01/05/2007

MARSH, FISCHMANN & BREYFOGLE LLP
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EXAMINER	
ROBERTS, LEZAH	
ART UNIT	PAPER NUMBER
1614	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
3 MONTHS	01/05/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

Office Action Summary	Application No.	Applicant(s)	
	10/728,277	ROSENTHAL ET AL.	
	Examiner	Art Unit	
	Lezah W. Roberts	1614	

-- *The MAILING DATE of this communication appears on the cover sheet with the correspondence address* --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *29 September 2006*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1,15,17,19,20,22,24,25,31,35,38,133-137 and 142-148* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1, 15, 17, 19-20, 22, 24-25, 31, 35, 38, 133-137 and 142-148* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *A-B*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

This office action is in response to the amendment filed September 29, 2006. All previous rejections have been withdrawn unless stated below.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action mailed March 23, 2006.

Response to Declaration Under 37 CFR 1.132

The Declaration of Janice M. Troha under 37 CFR 1.132 filed September 29, 2006 is insufficient to overcome the rejection of the instant claims based upon 35 USC 102 and 103 as set forth in the last Office action because: the Declaration shows methods of using the compositions. The claims are directed to a composition, however not a method of use. The intended use of a composition carries no weight in determining patentability because the compositions suggested by the references are substantially the same as the compositions of the instant claims.

Claims

Claim Rejections - 35 USC § 103 (Previous Rejection)

Claims 15, 22-23 and 136-141 were rejected under 35 U.S.C. 103(a) as being unpatentable over Krezanoski (US 4,188,373) in view of Boggs (US 5,358,705). The rejection is maintained in regards to claims 15, 22, 136-137 and 140.

Applicant argues Krezanoski does not disclose N-acetylcysteine (NAC), for any purpose. Applicant further argues based on the Troha Declaration, the disclosure of Boggs et al. would not lead to an expectation that NAC would be efficacious for treatment of mucositis occurring as a side effect of cancer therapy, the pathogenesis of which does not appear to be due to the presence of bacteria.

In response to applicant's argument that Krezanoski does not disclose NAC for any purpose and the disclosure of Boggs et al. would not lead to an expectation that NAC would be efficacious for treatment of mucositis occurring as a side effect of cancer therapy, the test for obviousness is not whether the features of a secondary reference may be bodily incorporated into the structure of the primary reference; nor is it that the claimed invention must be expressly suggested in any one or all of the references. Rather, the test is what the combined teachings of the references would have suggested to those of ordinary skill in the art. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981).

Claim Rejections - 35 USC § 103 – Obviousness (New Rejection)

Claims 1, 15, 19, 31, 35, 38, 133-137, 142-143 and 146 are rejected under 35 U.S.C. 103(a) as being unpatentable over Dobrozsi et al. (US 6,503,955).

Dobrozsi et al. disclose pourable liquid vehicles comprising an aqueous or nonaqueous polymer solution. The vehicles comprise a polyoxyalkylene block copolymer, water and glycols. The copolymer comprises polyoxypropylene and polyoxyethylene and makes up 25% to 77% by weight of the vehicle. Water makes up

5% to 45% of the composition (col. 7, lines 5-25). The glycols used, such as polyethylene glycol, which encompasses claim 11, make up 0 to 70% (col. 6, lines 50-53). Pluronic F-127 is a preferable block copolymer used in the compositions. The pourable liquid vehicle of the disclosed invention were formulated so that the contacting and mixing said vehicles to a mucosal surface of the body, or with some other fluid in the body, triggers the conversion of the pourable liquid vehicle to a more viscous gel-like mixture (col. 4, lines 33-48). The viscosities of the formulated vehicles were measured at room temperature and 37°C the temperature inside the human body. It was disclosed the viscosity of the compositions increased at the higher temperature, therefore encompassing claim 1. The disclosed liquid compositions have a viscosity of less than about 7 pascal seconds, preferably less than about 2 pascal seconds, more preferably less than about 1 pascal seconds (col. 5, lines 12-17), which encompasses no larger than 60cP of the instant claims. The desired value of a composition's triggered viscosity ratio is least about 1.3, preferably at least about 2, more preferably at least about 5, and most preferably at least about 10. The triggered viscosity is defined as the viscosity of the gel divided by the viscosity of the liquid. Using this calculation the gel viscosity is greater than 80cP, which encompasses the instant claims. The pourable liquid vehicles have a number of utilities including delivery of therapeutic agents. These include agents selected from the group consisting of expectorants/mucolytics, antioxidants and mixtures thereof (col. 7 lines 48-51). Expectorants/mucolytics include N-acetylcysteine. The active agents are added to the vehicles ranging up to 5% weight of the total composition according to the disclosed examples, which encompasses claim

15. The reference discloses several different dosage forms including gels, rinses, sprays and liquid filled capsules for intra-oral administration. Flavors and preservatives are also used in the disclosed compositions (see examples), as recited in claims 35 and 38.

The reference differs from the instant claims insofar as it does not disclose specifically using N-acetylcysteine in a composition comprising poloxamers 407. The reference is not anticipatory insofar as one must "pick and choose" from different lists of active agents and poloxamers. That being said, it would have been obvious in a self-evident manner to have selected N-acetylcysteine from one list and poloxamers 407 from another, motivated by the unambiguous disclosure of each individually, and consistent with the basic principle of patent prosecution that a reference should be considered as expansively as is reasonable in determining the full scope of the contents within its four corners.

2) Claims 17, 20, 24-25 and 137, 140 144-145 and 147-148 are rejected under 35 U.S.C. 103(a) as being unpatentable over Dobrozsi et al. (US 6,503,955) in view of Stratton et al. (US 5,861,174).

The primary reference is discussed in subsection 1, above. The reference differs from the instant claims insofar as it does not disclose the compositions comprise 0.1 to 20% of the preferred block copolymer, the compositions comprise about 10% or N-acetylcysteine and the composition were made when the liquid carrier was 5°C.

Stratton et al. disclose pharmaceutical compositions for the delivery of pharmacologically active proteins. The polypeptides make up 0.5% or greater of the disclosed compositions (col. 3, lines 38-45). In one embodiment of the invention, the polypeptide comprises 0.5 to 50% by weight of the compositions (col. 6, lines 46-50). The polymers of disclosed invention provide a sustained release delivery system for active agents or drugs (col. 1, lines 51-53) The delivery vehicle comprises block copolymers, polyoxyethylene-polyoxypropylene, namely Pluronic polyols, or poloxamers. Poloxamers have the ability to gel as a function of temperature and polymer concentration. Poloxamers having molecular weights below 10,000, do not form gels at any concentration, therefore Pluronic F-127 and Poloxamer 407 are the polymers of choice for the disclosed invention (col. 2, lines 18-60). These polymers have the characteristics of being liquid at temperatures below room temperature but will form a gel as they are warmed (col. 4, lines 38-41). The aqueous polymer solutions may be formed in two ways, by a cold process or by a hot process. The cold process involves dissolving the polymer at a temperature from about 5°C to 10°C (col. 5, lines 20-34). When adding the polypeptide, it is preferred to add the agent at a temperature of about 0°C to 10°C. These conditions encompass claims 24-25. Raising the sample temperature above the gel point of the poloxamer results in an even distribution of protein particles throughout the polymer gel (col 6, lines 1-7). The copolymer will not form a gel at a concentration outside the range of about 20% to 30% by weight (which overlaps the concentration of the instant claims) but it was discovered other compounds could be added to the compositions in order for the copolymer to form a gel

at concentrations lower than 20% by weight, which encompasses claim 137 as well as claim 20.

The reference differs from the instant claims insofar as it does not disclose compositions comprising glutathione or its precursors and the viscosities of the compositions before and after the temperature change.

It would have been obvious to adjust the amount of poloxamer in the compositions of the primary reference motivated by the desire to obtain the desired characteristics of the composition, such as the removal of the reverse-thermal gelation property as recited in claim 20, as disclosed by the secondary reference.

It would also have been obvious to one of ordinary skill in the art to have used the delivery system comprising 20 to 30 percent poloxamer and theory to deliver the active agents of the primary reference motivated by the desire to provide a sustained release composition that exist in a liquid form and gels when introduced into the body wherein the therapeutic composition is released over a period of time, as disclosed by the secondary reference.

Normally, changes in result effective variables are not patentable where the difference involved is one of degree, not of kind experimentation to find workable conditions generally involves the application of no more than routine skill in the art. In re Aller 105 USPQ 233, 235 (CCPA 1955). It would also have been obvious to one of ordinary skill in the art to have adjusted the amount of N-acetylcysteine in the compositions of the primary reference motivated by the desire to deliver an effective amount of active agent to obtain optimal results as supported by cited precedent.

3) Claims 1, 15, 20, 22, 24-25, 35, 38, 137, 140 and 142-148 are rejected under 35 U.S.C. 103(a) as being unpatentable over Boggs (US 5,358,705) in view of Stratton et al. (US 5,861,174).

Boggs et al. disclose oral compositions for preventing conditions of the oral cavity. The active ingredients in the compositions include N-acetylcysteine complexes, which make up 0.05 to 10% of the compositions as recited in the instant claims. These concentrations are considered "safe and effective", which is defined as an amount of compound or composition sufficient to induce a significant positive modification in the condition being treated, but low enough to avoid serious side effects (col. 4, lines 11-32). The compositions also include surfactants such as Pluronic F-127 and make up 0 to 10% of the compositions. The reference differs from the instant claims insofar as it does not specifically disclose the compositions exhibit thermal-reversible behavior.

The secondary reference is discussed above and disclosed the thermal properties of polyoxyethylene and polyoxypropylene copolymers. It is used as a general teaching to show the surfactants used in the compositions of the primary reference are thermal responsive polymers and do not display thermal responsive gelation at the disclosed concentrations. The reference differs from the instant claims insofar as it does not disclose comprising N-acetylcysteine in the compositions.

It would have been obvious to one of ordinary skill it the art to have used the amounts of poloxamer used in the compositions of the primary reference motivated by the desire to inhibit gel formation but still has an increased viscosity when introduced

into the body to prolong the release of the active agent, as disclosed by the secondary reference.

Claims 1, 15, 17, 19-20, 22, 24-25. 31, 3[illegible], 38, 133-137 and 142-148 are rejected.

No claims allowed.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lezah W. Roberts whose telephone number is 571-272-1071. The examiner can normally be reached on 8:30 - 5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ardin H. Marschel can be reached on 571-272-0718. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Lezah Roberts
Patent Examiner
Art Unit 1614

Frederick Krass
Primary Examiner
Art Unit 1614

APPENDIX C

TO

DECLARATION

OF

ANTONY JAMES MATHEWS

Copy:

Dobrozsi et al. (US Patent No. 6,503,955)

APPENDIX D

TO

DECLARATION

OF

ANTONY JAMES MATHEWS

Copy of Claims Considered

1. A therapeutic composition useful for treatment of oral mucositis as a side effect of cancer therapy, the composition comprising:

N-acetylcysteine in an amount effective as formulated in the composition to provide therapeutic effect for treatment of the mucositis;

from 5 weight percent to 20 weight percent poloxamer 407;

a carrier liquid comprising water in an amount sufficient as formulated in the composition to interact with the poloxamer 407 to impart reverse-thermal viscosity behavior to the therapeutic composition, wherein the composition exhibits the reverse-thermal viscosity behavior over at least some range of temperatures between 1°C and 37°C;

wherein, at some temperature in a range of from 2°C to 8°C the therapeutic composition is in the form of an aqueous solution with the poloxamer 407 and the N-acetylcysteine dissolved in the water.

15. The therapeutic composition of Claim 1, wherein the N-acetylcysteine comprises from about 0.001 percent by weight to about 50 percent by weight of the composition.

17. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits the reverse-thermal viscosity behavior over at least some range of temperatures between 1°C to 20°C.

19. The therapeutic composition of Claim 1, wherein the biocompatible polymer, as formulated in the therapeutic composition, imparts a reverse-thermal gelation property to the composition with the composition having a reverse-thermal liquid-gel transition temperature within a range of from 1°C to 37°C, so that the therapeutic composition gels as the temperature of the therapeutic composition is increased from below to above the reverse-thermal gel transition temperature.

20. (Previously Presented) The therapeutic composition of Claim 1, wherein the amount of the water, as formulated in the composition, does not interact with the poloxamer 407 to impart reverse-thermal gelation properties to the composition.

22. The therapeutic composition of Claim 1, wherein the poloxamer 407 comprises from 5 weight percent to 20 weight percent of the composition.

24. The therapeutic composition of Claim 1, wherein the poloxamer 407 is dissolved in the water when the composition is at a temperature of 5°C.

25. The therapeutic composition of Claim 24, wherein the N-acetylcysteine is dissolved in the water when the composition is at a temperature of 5°C.

31. The therapeutic composition of Claim 1, comprising a bioadhesive agent that is different than the N-acetylcysteine and the poloxamer 407.

35. The therapeutic composition of Claim 1, comprising at least one taste masking component.

38. The therapeutic composition of Claim 1, comprising at least one preservative component.

133. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits an increase in viscosity from no larger than about 60cP to at least about 70cP when a temperature of the composition is increased from 1°C to 37°C.

134. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits an increase in viscosity from no larger than about 60cP to at least about 80cP when a temperature of the composition is increased from 1°C to 37°C.

135. The therapeutic composition of Claim 1, wherein the therapeutic composition exhibits an increase in viscosity from no larger than about 50cP to at least about 70cP when a temperature of the composition is increased from 1°C to 37°C.

136. The therapeutic composition of Claim 1, wherein the composition comprises reverse-thermal gelation properties with a reverse-thermal liquid-gel transition temperature within the range of temperatures.

137. The therapeutic composition of Claim 1, wherein the therapeutic composition comprises from 0.1 to 20 weight percent of the N-acetylcysteine.

140. The method of Claim 137, wherein the therapeutic composition comprises about 10 weight percent of the N-acetylcysteine.

142. The therapeutic composition of Claim 1, wherein:

the therapeutic composition is adapted for delivery to a patient when the therapeutic composition is at a refrigerated temperature in a range of from 1°C to 10°C; and

when the therapeutic composition is at the refrigerated temperature, it is in the form of a flowable medium with each of the N-acetylcysteine and the poloxamer 407 dissolved in the water.

143. The therapeutic composition of Claim 142, comprising from 0.1 weight percent to 25 weight percent of the N-acetylcysteine.

145. The therapeutic composition of Claim 143, comprising from 10 weight percent to 20 weight percent of the poloxamer 407.

146. The therapeutic composition of Claim 143, comprising up to 10 weight percent of the N-acetylcysteine.

147. The therapeutic composition of Claim 143, comprising about 10 weight percent of the N-acetylcysteine.

148. The therapeutic composition of Claim 147, comprising from 10 weight percent to 20 weight percent of the poloxamer 407.

149 The therapeutic composition of Claim 143, wherein when the therapeutic composition is at a temperature of 2°C the therapeutic composition has sufficient fluidity for use as a mouthwash that can be swished in the oral cavity.

150. The therapeutic composition of Claim 143, wherein when the therapeutic composition is at a temperature of 2°C the viscosity of the therapeutic composition is no larger than 60 cP.

151. The therapeutic composition of Claim 143, wherein the carrier liquid is water.

152. The therapeutic composition of Claim 143, wherein the carrier liquid comprises, in addition to the water, at least one component selected from the group consisting of ethanol and a polyol.

APPENDIX E

TO

DECLARATION

OF

ANTONY JAMES MATHEWS

Summary Of Viscosity Tests On Example Compositions

Example 1: Viscosity Behaviors of Formulations Comprising 26% Lutrol® F127, 50% water and 24% Ethanol:

Two 100 gram formulations with the same composition of Lutrol® F127: ethanol: water (26:24:50 w/w) were prepared by the different methods as described below. Lutrol® F127 is a registered trademark of BASF Corporation, and is a pharmaceutical grade of Pluronic F127, a poloxamer 407.

Preparation of Formulation #1: 26g of Lutrol® F127 (Prill, National Formulary pharmaceutical grade, BASF), 24g ethanol (200 Proof anhydrous, 99.5%, Acros) and 50g water (RO water, 18 Megaohm.cm^{-1} resistance) were added to a bottle and then cooled on ice. Additionally, the formulation was mixed vigorously with a magnetic stirrer and stir bar, and by shaking the bottle. This formulation became homogenous within a few minutes of mixing.

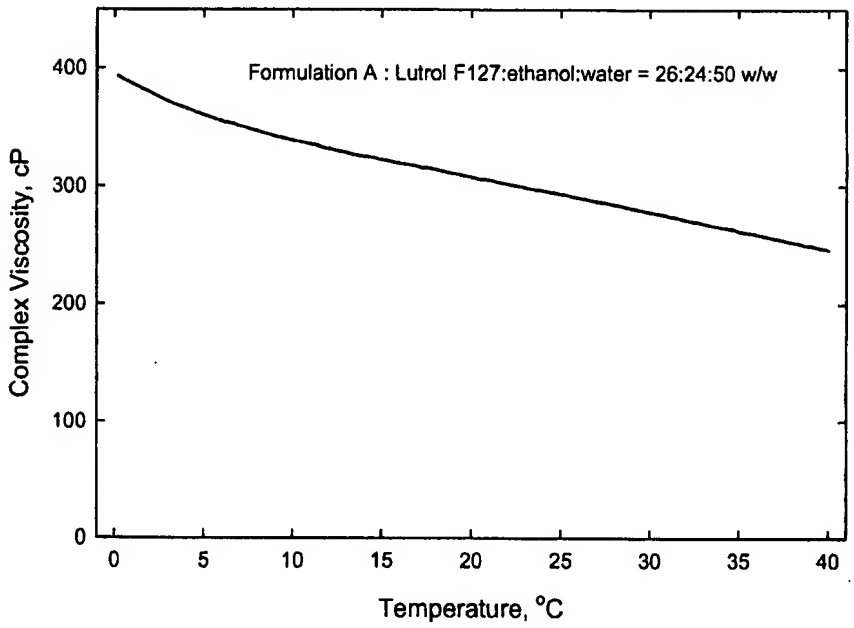
Preparation of Formulation #2: A stock solution of 34.2% w/w Lutrol® F127 (Prill, National Formulary pharmaceutical grade, BASF) was prepared in water (RO water, 18 Megaohm resistance). To prepare this stock solution, 1316g of water was cooled on ice and then 684g Lutrol® F127 was added with mixing by an overhead mechanical stirrer for 2 hours. After mixing, the stock solution was stored at 4 °C overnight to allow the foam to break down. This process yields approximately 2000g of stock solution. 76g of this stock solution thus contained 26 g of Lutrol® F127 and 50g of water.

Formulation 2 was then prepared by adding 24g of ethanol to 76g of the cold stock solution. The composition was mixed using a magnetic stirrer and stir bar. The formulation immediately formed a homogeneous solution, and was stored at 4 °C.

Analysis: Both formulations 1 and 2 appeared physically identical regardless of their method of preparation and are collectively referred to as Formulation A for the following rheological analysis. The analysis was performed using TA Instruments AR500 Rheometer. A temperature ramp oscillation experiment was implemented to observe viscosity behavior as a function of temperature from 0 to 40 °C.

Results: Figure 1 illustrates thermal viscosity behavior measured for Formulation A between 0 °C and 40 °C. Formulation A does not exhibit reverse-thermal viscosity behavior between 0°C and 40 °C. Figure 1 demonstrates that the viscosity of Formulation A steadily decreased from 400cP at 0 °C to 250cP at 40 °C.

Figure 1: Viscosity Behavior of Formulation A



Example 2: Viscosity Behaviors of Formulations Comprising 26% Lutrol® F127, 50% water and 24% Propylene Glycol:

Two 100 gram formulations with the same composition of Lutrol® F127: propylene glycol: water (26:24:50 w/w) were prepared by the different methods as described below. Lutrol® F127 is a registered trademark of BASF Corporation, and is a pharmaceutical grade of Pluronic F127, a poloxamer 407.

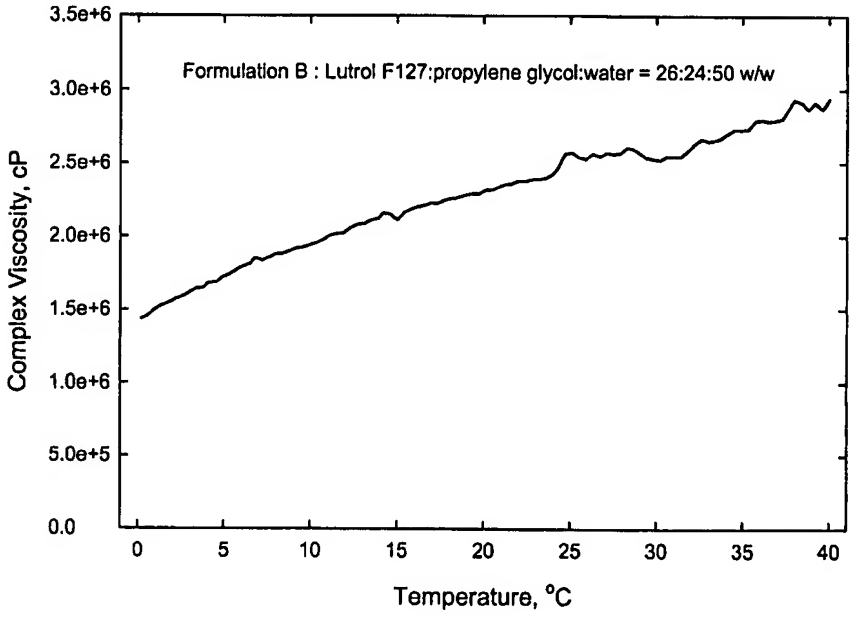
Preparation of Formulation #3: 26g of Lutrol® F127 (Prill, National Formulary pharmaceutical grade, BASF), 24g propylene glycol (USP grade, Fisher) and 50g water (RO water, 18 Megaohm.cm^{-1} resistance) were added to a bottle and then cooled on ice. Additionally, the formulation was mixed vigorously with a magnetic stirrer and stir bar, and by shaking the bottle. This formulation required storage at -20 °C for 48 hours before becoming homogenous.

Preparation of Formulation #4: A stock solution of 34.2% w/w Lutrol® F127 (National Formulary pharmaceutical grade, BASF) was prepared in water (RO water, 18 Megaohm.cm^{-1} resistance) as described in Example 1. 76g of the stock solution thus contained 26g of Lutrol® F127 and 50g water. Formulation 4 was then prepared by adding 24g of propylene glycol to 76g of the cold stock solution. The formulation was mixed using a magnetic stirrer and stir bar. Formulation 4 required storage overnight at -20°C before it became homogenous.

Analysis: Both formulations 3 and 4 appeared physically identical regardless of their method of preparation and are collectively referred to as Formulation B for the following rheological analysis. Analysis was performed using TA Instruments AR500 Rheometer. A temperature ramp oscillation experiment was implemented to observe viscosity behavior as a function of temperature from 0 to 40 °C.

Results: Figure 2 illustrates thermal viscosity behavior measured for Formulation B between 0 °C and 40 °C. The viscosity for Formulation B did exhibit reverse-thermal viscosity behavior, with the viscosity of the composition increasing from 1,400,000cP at 0 °C to 3,200,000cP at 40°C. Figure 2 illustrates that at 25°C the viscosity of Formulation B is approximately 2,500,000cP, or about 350 times greater than the maximum viscosity of 7000cP permitted by Dobrozsi et al. for the compositions of their pourable liquid vehicle.

Figure 2: Viscosity Behavior of Formulation B



Example 3: Viscosity Behavior of a Formulation Comprising 26% Lutrol® F127, 37.1% water and 36.9% Ethanol:

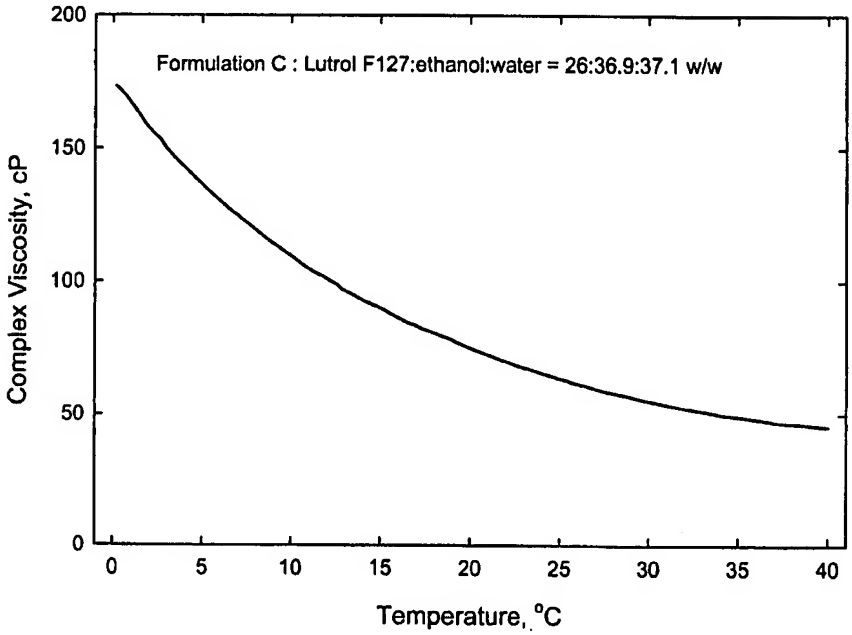
A 100 gram formulation with the composition of Lutrol® F127: ethanol: water (26:36.9:37.1 w/w) was prepared as described below. Lutrol® F127 is a registered trademark of BASF Corporation, and is a pharmaceutical grade of Pluronic F127, a poloxamer 407.

Preparation of Formulation #5: 26g of Lutrol® F127 (Prill, National Formulary pharmaceutical grade, BASF), 36.9g ethanol (200 Proof anhydrous, 99.5%, Acros) and 37.1g water (RO water, 18 Megaohm.cm^{-1} resistance) were added to a bottle and then cooled on ice. Additionally, the formulation was mixed vigorously with a magnetic stirrer and stir bar, and by shaking the bottle. This formulation became a clear homogenous solution within a few minutes of mixing and was stored at 4°C.

Analysis: The rheological analysis of this formulation was performed using a TA Instruments AR500 Rheometer. A temperature ramp oscillation experiment was implemented to observe viscosity behavior as a function of temperature from 0 to 40 °C. For rheological analysis, this formulation is referred to as Formulation C.

Results: Figure 3 illustrates the thermal viscosity behavior measured for Formulation C between 0 °C and 40 °C. Formulation C does not exhibit reverse-thermal viscosity behavior between 0°C and 40°C. Figure 3 demonstrates that the viscosity of Formulation C decreased steadily from 175cP at 0 °C to ca. 50cP at 40 °C.

Figure 3: Viscosity Behavior of Formulation C



Example 4: Viscosity Behavior of a Formulation Comprising 26% Lutrol® F127, 37.1% water and 36.9% Propylene Glycol:

Two 100 gram formulations were prepared with the composition of Lutrol® F127: propylene glycol: water (26:36.9:37.1 w/w) by the different methods as described below. Lutrol® F127 is a registered trademark of BASF Corporation, and is a pharmaceutical grade of Pluronic F127, a poloxamer 407.

Preparation of Formulation #6: 26g of Lutrol® F127 (Prill, National Formulary pharmaceutical grade, BASF), 36.9g propylene glycol (USP grade, Fisher) and 37.1g water (RO water, 18 Megaohm.cm^{-1} resistance) were added to a bottle and then cooled on ice. Additionally, the formulation was mixed vigorously with a magnetic stirrer and stir bar, and by shaking the bottle. This formulation was stored at -20 °C for at least 2 weeks with occasional vigorous mixing, but never became homogeneous and large particles of Lutrol® F127 remained apparent.

Preparation of Formulation #7: 26g of Lutrol® F127 (Prill, National Formulary pharmaceutical grade, BASF) and 37.1g water (RO water, 18 Megaohm.cm^{-1} resistance) were added to a bottle and then cooled using an ice/salt bath. These formulation components were mixed vigorously using a Silverson L4RTA high shear laboratory mixer equipped with a 3/4" inch tubular mixing assembly and containing a square hole high shear screen. Various mixing speeds were used up to 4,000rpm. After 5 minutes of mixing, 36.9g of propylene glycol was added with vigorous mixing and the formulation was mixed for an additional 10 minutes and then stored at -20°C. This formulation did not become homogeneous within 1 week of storage at -20°C, and a significant amount of solid Lutrol® F127 remained.

Analysis: Both formulations 6 and 7 appeared physically very similar regardless of their method of preparation and are collectively referred to as Formulation D for the following comments. Analysis of Formulation D was not attempted because it failed to form a homogeneous state.